

**THE EFFECTS OF COMBUSTION DERIVED AIR POLLUTION ON
VASCULAR AND FIBRINOLYTIC FUNCTION IN MAN**

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To Charlie

ABSTRACT

Observational studies have consistently demonstrated associations between exposure to air pollution and increased cardiovascular morbidity and mortality. These associations are strongest for fine particulate matter (PM), of which particulates from the combustion of fossil fuels are an important component. In Europe, the contribution to urban PM from diesel emissions is increasing with the popularity of diesel engines for road transport. Despite the strength of the epidemiological evidence and the emergence of promising hypotheses, the important constituents and biological mechanisms responsible for the cardiovascular effects of air pollution are largely unknown.

It is possible that nanoparticulates or soluble components of PM may translocate into the bloodstream, resulting in direct effects on the vascular endothelium and thrombotic pathways. I investigated the potential for inhaled radiolabelled nanoparticulates to translocate into the circulation in man. Using two unique human exposure facilities I assessed the effects of exposure to combustion-derived particulates in dilute diesel exhaust and concentrated ambient fine and ultrafine particles on vascular endothelial, endogenous fibrinolytic and myocardial function in healthy volunteers and patients with stable coronary artery disease.

In total, forty-two healthy men and thirty-two patients with stable coronary artery disease were exposed to particulates or filtered air for 1-2 hours in a series of double blind randomised crossover studies. At levels encountered in an urban environment, inhalation of dilute diesel exhaust impaired two important and complementary

aspects of vascular function in man: the regulation of vascular tone and endogenous fibrinolysis. Vascular dysfunction persisted for up to 24-hours following exposure and was associated with an increase in systemic inflammatory cytokines. In patients with coronary heart disease exposure to diesel exhaust did not aggravate pre-existing vasomotor dysfunction, but did exacerbate exercise-induced myocardial ischemia and reduce acute endothelial tissue plasminogen activator release.

In contrast, exposure to concentrated ambient particulates, low in combustion component, did not affect vascular function in either healthy volunteers or patients. I found little evidence that inhaled radiolabelled nanoparticles translocate into the circulation and suggest the adverse vascular effects of combustion derived air pollution are mediated primarily by their soluble components rather than by a direct interaction between nanoparticles and the vasculature.

My findings have identified ischemic, vascular and thrombotic mechanisms that may explain in part the observations that exposure to combustion-derived air pollution is associated with adverse cardiovascular events including acute myocardial infarction. Ongoing research in this area will provide further insight into the adverse effects of PM, with the possibility of targeted interventions, such as the use of retrofit particle traps on diesel powered vehicles, to reduce the impact of environmental air pollution on cardiovascular disease a realistic goal.

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DECLARATION

This thesis represents research undertaken in the Centre for Cardiovascular Sciences, University of Edinburgh, the Department of Cardiology, Royal Infirmary of Edinburgh, and the Department of Respiratory and Allergy Medicine, Umeå University, Sweden during the period August 2003 to August 2006.

British Heart Foundation Project (03/017/15071) and Programme Grants (PG/05/003) sponsored these studies, which were conducted through a successful collaboration with the University of Umeå. I was personally involved in all of the exposures, vascular assessments and data analysis presented in the thesis. In keeping with the nature of collaborative research, assistance with the studies in Sweden was gratefully received from my colleagues Dr Håkan Törnqvist and Dr Manuel Gonzalez at Umeå University. Dr Mark Miller, Edinburgh University, undertook the electron-paramagnetic resonance studies presented in Chapter 4. Assistance was also provided in the supervision of the exposures and laboratory assays as acknowledged.

Chapters 1, 3, 4, 5, 6 and 7 have been published in peer-reviewed journals. I have copyright permission for inclusion of the printed journal manuscripts within this thesis. The thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged. All studies were undertaken in accordance with the regulations of the Regional Ethics Board of Umeå, the Lothian Research Ethics Committee and with the Declaration of Helsinki of the World Medical Association. The written informed consent of each subject or patient was obtained before entry into the study.

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ABBREVIATIONS

ACE = angiotensin converting enzyme
AHA = American Heart Association
ANOVA = analysis of variance
ATOFMS = aerosol time-of-flight mass spectrometer
AUC = area under curve
CAPs = concentrated ambient particles
CO = carbon monoxide
COMEAP = Committee of the Medical Effects of Air Pollution
CPC = condensation particle counter
CPM = counts per minute
CRP = C-reactive protein
DEP = diesel exhaust particles
EBC = exhaled breath condensate
EPA = Environmental Protection Agency
EPR = electron paramagnetic resonance
ET = endothelin
FBF = forearm blood flow
IL = interleukin
ICAM = inter-cellular adhesion molecules
MAPCEL = mobile ambient particle concentrator exposure laboratory
NAAQS = National Ambient Air Quality Standards
NO = nitric oxide
NO₂ = nitrogen dioxide
NO_x = nitrogen oxides
PAH = polycyclic aromatic hydrocarbons
PAI = plasminogen activator inhibitor
PM = particulate matter
PM_{2.5} = particulate matter less than 2.5µm in diameter
PM₁₀ = particulate matter less than 10µm in diameter
SEM = standard error of the mean
SMPS = scanning mobility particle sizer
SNP = sodium nitroprusside
SO₂ = sulphur dioxide
SOD = superoxide dismutase
TEAC = Trolox equivalent antioxidant capacity
TLC = thin layer chromatography
TNF-α = tissue necrosis factor alpha
t-PA = tissue plasminogen activator
VACES = Versatile ambient concentrator exposure system
WHO = World Health Organisation

CHAPTER 1

INTRODUCTION – AIR POLLUTION AND ATHEROTHROMBOSIS

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1.1 OVERVIEW

Observational studies have consistently demonstrated an association between exposure to particulate air pollution and increased cardiovascular morbidity and mortality. Despite the strength of the epidemiological evidence and the emergence of promising hypotheses, the important constituents and biological mechanisms responsible for the cardiovascular effects of air pollution are largely unknown. Oxidative stress and inflammation are central to both the toxicology of particulate air pollution and the pathogenesis of atherothrombosis. It is possible that nanoparticles or soluble components may translocate into the bloodstream, resulting in direct effects on atherosclerotic plaque stability, the vascular endothelium, platelet function and thrombosis. Research in this area will provide insight into the adverse vascular effects of air pollution, with the possibility of interventions to reduce the impact of environmental air pollution on cardiovascular disease a realistic goal.

1.2 AIR POLLUTION AND CARDIOVASCULAR RISK

Evidence accumulated over more than 50 years of epidemiological and clinical research has established the adverse effects of air pollution on human health. The London smog of December 1952 caused more than 4,000 excess deaths [Ministry of Health, 1954], and despite the dramatic decreases in levels of air pollution that have been achieved since then, the association between air pollution and cardiorespiratory morbidity and mortality persists and has been widely established [Anderson *et al*, 1996; Dockery *et al*, 1993]. The World Health Organisation (WHO) currently estimates three million people die each year of air pollution, representing 5% of the 55 million deaths occurring annually in the world [WHO Statistics].

Short-term increases in air pollution exacerbate existing cardiorespiratory disease leading to increased hospital admissions and in some patients, death [Peters *et al*, 2001a]. Cardiovascular events are now thought to be responsible for the majority of these excess deaths [Pope, 2000]. Recent work from several USA cities indicated each $10 \mu\text{g}/\text{m}^3$ elevation in fine particles was associated with a 4%, 6% and 8% increase in the risk of all cause, cardiorespiratory and lung cancer mortality respectively [Pope *et al*, 2002]. In Edinburgh, a city with relatively low levels of pollution, an increase in particulate matter (PM) of $10 \mu\text{g}/\text{m}^3$ was associated with an increase in emergency cardiovascular admissions of 4.8% [Prescott *et al*, 1998]. Exposure to air pollution has been associated with exercise-induced myocardial ischaemia in patients with coronary heart disease [Pekkanen *et al*, 2002] and the triggering of acute myocardial infarction [Peters *et al*, 2001a]. In concordance with these short-term effects, the risk of mortality from cardiovascular disease is greater

for those living in areas of greater pollution [Dockery *et al*, 1993; Pope *et al*, 2002]. In the most comprehensive study to date Miller and colleagues estimate that long-term exposure to air pollution increases the risk of death from cardiovascular disease by as much as 76% [Miller *et al*, 2007]. In the UK the government has estimated that around 8,000 excess deaths occur per annum as a result of air pollution.

A recent Scientific Statement by the American Heart Association (AHA) [Brook *et al*, 2004] acknowledges that exposure to air pollution is an important risk factor for the development of cardiovascular disease. Whilst it is possible to derive estimates of the magnitude of risk associated with a population exposure over many years it is difficult to estimate individual risk and therefore most governmental advisory bodies do not advocate restricting activity on high air pollution days. However, in the recent AHA statement the authors suggest that patients with cardiovascular risk factors or existing disease should curtail physical activity when air pollution levels exceed Air Quality Standards. Perhaps a surprising statement, given at the time of writing there had been no published studies to assess the direct effects of air pollutants on patients with ischaemic heart disease. The Committee on the Medical Effects of Air Pollution (COMEAP) who advise the Department of Health in the UK have recently reviewed the literature and concluded that patients should not avoid exercise on the grounds that this will reduce their exposure to air pollutants as the value of exercise in preventing heart disease is likely to be more important [Committee on the Medical Effects of Air Pollution, 2006].

Both COMEAP and the AHA Expert Panel agree that a better understanding of the components and mechanisms responsible for the association between air pollution and cardiovascular disease is urgently needed.

1.3 THE ROLE OF PARTICULATE AIR POLLUTION

Air pollutants implicated as potentially harmful include particles, nitrogen dioxide, ozone, sulphur dioxide and volatile organic compounds and the particles appear to be the most harmful component of the pollution 'cocktail'. Large particles are mostly derived from soil and crustal elements whereas fine particles are primarily produced from the combustion of fossil fuels by motor vehicles or in power generation. Both the WHO and United Nations have declared that the most significant global air pollution threat is posed by PM. Of these, only small particles can be inhaled into the lungs and national air standards have been based on the mass concentration of such 'inhalable' particles, typically defined as having an aerodynamic diameter centred around 10 μm (PM_{10}). In the world's twenty largest cities, peak concentrations of PM_{10} may exceed 1000 $\mu\text{g}/\text{m}^3$, with average levels in the range 200-600 $\mu\text{g}/\text{m}^3$ and only three of these cities have levels of PM pollution within current guidelines [WHO Report, 1994].

The individual components of PM_{10} are not especially toxic at ambient levels and some that contribute a large part of the mass, e.g. salt crystals, are harmless.

Therefore toxicologists generally consider that one or more components of PM_{10} mediate the adverse health effects. The main components considered likely initiators of inflammation leading to the adverse effects of PM_{10} are combustion-derived nanoparticles, transition metals, and polycyclic aromatic hydrocarbons (PAH).

Pope *et al* demonstrated the risk of adverse effects associated with $\text{PM}_{2.5}$ (particles <2.5 μm in aerodynamic diameter) was greater than for PM_{10} [Pope *et al*, 2002] and

toxicological studies have emphasised the increased toxicity of the smaller size fractions [reviewed in Donaldson *et al*, 2000; Donaldson *et al*, 2001]. PM₁₀ contains a variable ultrafine component that is greatest near automotive sources. Diesel soot is an example of a carbon-centred combustion-derived particle that is ultrafine in terms of primary particle size [Tobias *et al*, 2001]. It has been hypothesised that ultrafine particles show more free radical oxidant capacity than non-ultrafine particles suggestive of a greater direct oxidative stress at the particle surface [Li *et al*, 1999]. Thus the toxicity of particulate matter appears to relate to the number of particles encountered, their size or surface area, and their chemical composition [MacNee and Donaldson, 2000]. The very small particles (<50 nm) derived directly from combustion sources aggregate readily into larger aggregates, ranging from nanometre size up to a micrometer. It is unclear if these aggregates of ultrafine particles in PM₁₀, although not ultrafine by aerodynamic size, maintain the toxicity of their ultrafine components. However, in toxicological studies, aggregates of such particles cause lung responses consistent with their surface area predicted from the ultrafine particle and not their geometry [Duffin *et al*, 2002].

1.4 OBSERVATIONAL STUDIES

The cardiovascular morbidity and mortality associated with increases in PM are well documented [reviewed in Brook *et al*, 2003; Brook *et al*, 2004; Routledge *et al*, 2003]. Temporal variations in PM are utilised by ‘time series’ studies to relate the moving average to defined end-points such as hospital admissions for ischaemic heart disease or cardiovascular deaths. Using this approach there is a clear relationship between PM₁₀ and cardiovascular events in the days following an increase in the levels of particulate (Table 1.1).

Table 1.1. Percentage change in cardiovascular outcomes per 10µg/m³ increase in PM₁₀ (Adapted from the COMEAP Report, 2006)

Outcome	Percentage change per 10 µg/m ³
Cardiovascular mortality	0.5 (0.4-0.7)
Cardiovascular admissions	0.5 (0.2-0.7)
Ischaemic heart disease admissions	0.8 (0.6-0.9)

The composition of urban PM is dependent on local sources, with concentrations dependent on a number of variables including distance from source, wind speed and direction. This spatial dimension is utilised in environmental studies where populations living in areas of high air pollution are compared to those in areas with low air pollution. A good example of this type of study is the Harvard 6 cities study,

where cardiovascular disease was more prevalent in areas with the highest PM levels (Table 1.2).

Table 1.2. Adjusted mortality rate ratios for the most versus the least polluted city in the US 6-cities study (Adapted from the COMEAP Report, 2006)

Outcome	Most versus least polluted city
All mortality	1.26 (1.08-1.47)
Mortality due to cardiopulmonary disease	1.37 (1.11-1.68)

More detailed information, helpful in understanding the likely mechanisms responsible for the cardiovascular effects of PM, has been provided by panel studies. These studies relate changes in physiological endpoints to temporal changes in PM air pollution in susceptible cohorts. Elevations in PM have been associated with increases in heart rate [Peters *et al*, 1999], decreased heart rate variability [Gold *et al*, 2000], and systemic vascular dysfunction [O'Neill *et al*, 2005]. Further discussion of these observational studies can be found in the following useful and detailed reviews of the epidemiological literature [Pope, 2000; Brunekreef and Holgate, 2002].

1.5 HUMAN EXPOSURES SYSTEMS

Epidemiological studies cannot prove a causative biological effect of air pollution exposure. Assessing individual human exposure to particles using data from local area monitoring systems may be inaccurate as it is known that personal exposures may be several times higher than corresponding area measurements [Watt *et al*, 1995]. Systems designed to deliver controlled amounts of air pollution now exist to allow a mechanistic approach to determining the effect of inhaled PM. These include concentrated ambient particles (CAPs) within filtered air [Ghio *et al*, 2000] or controlled delivery of diesel exhaust particles [Nightingale *et al*, 2000; Salvi *et al*, 1999].

There are significant differences in the chemical composition and size distribution of PM at various distances from a road because of a wide range of sources, meteorological conditions, atmospheric chemistry, and temporal factors. The development of PM concentrators has fundamentally changed research into the causal factors associated with exposure to PM. The concentrators are capable of concentrating particles up to 30 times ambient concentrations thereby facilitating toxicological studies which were previously impossible, and allowing researchers to accurately quantify dose-response relationships following PM exposure.

Because of the hypothesis that combustion-derived particles are important, studies utilising diesel exhaust remain crucial in determining the health effects of exposure to the combustion-derived component of air pollution [Nightingale *et al*, 2000;

Rudell *et al*, 1994]. Compared with petrol engines, diesel engines produce less carbon monoxide but more nitrogen oxides and aldehydes. They also generate an ultrafine singlet particulate that is carbon-centred, and polycyclic aromatic hydrocarbon, metal and sulphate-rich [Scheepers and Bos, 1992]. These rapidly aggregate and are thought to be one of the key components that mediate the observed adverse health effects of PM₁₀ [Schwartz *et al*, 1996]. The particulate emission from diesel engines is over 100 times higher than the particulate emission from an equivalent petrol engine with a catalytic converter [Sydbom *et al*, 2001]. Over 80% of diesel exhaust particles have a size of <0.1 µm, and these represent a substantial component of the PM₁₀ fraction. By way of their larger surface area, these submicron particles may carry a large fraction of toxic compounds on their surface [Levsen, 2002]. They are deposited deep within the lung with high efficiency from where they are only slowly cleared leading to a prolonged time period over which they might exert harmful effects.

1.6 POTENTIAL MECHANISMS

A number of interesting theories have been proposed to explain the association between increased PM and cardiovascular disease. Based on our existing knowledge, we present a summary of the potential pathways linking inhaled particles to atherothrombosis and adverse cardiovascular outcomes in Figure 1.1. According to this model, inhaled particles cause pulmonary and systemic inflammation that indirectly impacts on the cardiovascular system. Smaller particles may also translocate into the bloodstream exerting direct effects on the vasculature. Once in the circulation particles could affect thrombogenesis through endothelial cell or platelet activation, or may penetrate the vessel wall, promoting atherosclerosis or predisposing to plaque rupture.

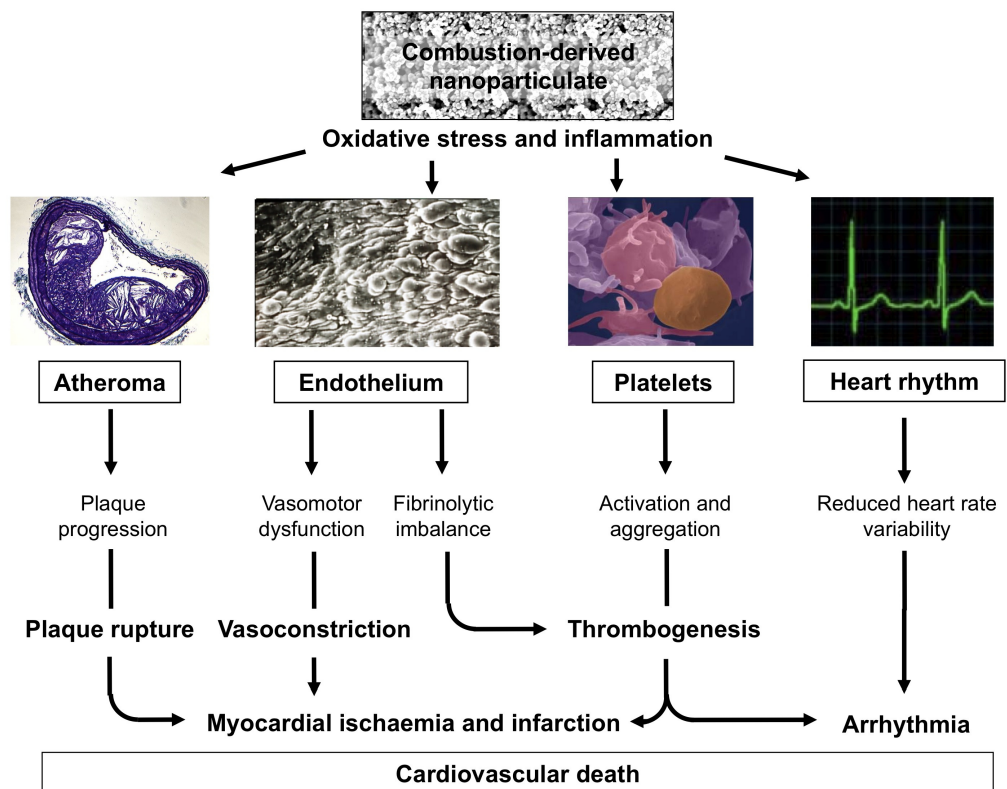


Figure 1.1 Hypothetical pathways linking inhaled particles to the pathogenesis of atherothrombosis and acute cardiovascular events.

1.6.1 Pulmonary and Systemic Inflammation

In vitro studies, animal models and human exposures have clearly established that particles exert their pathogenic effects primarily through oxidative stress and inflammation [Donaldson and Tran, 2002]. PM₁₀ particles cause lung inflammation in animal models following intra-pulmonary instillation [Li *et al*, 1996] and inhalation of roadside ambient particles [Elder *et al*, 2004a]. In human studies inflammatory effects have been demonstrated following inhalation of CAPs [Ghio *et al*, 2000; Holgate *et al*, 2003] and dilute diesel exhaust [Salvi *et al*, 1999; Salvi *et al*, 2000].

Increases in plasma or serum markers of systemic inflammation have been reported following exposure to particles. In panel and population studies PM exposure is associated with evidence of an acute phase response with increased C-reactive protein (CRP) [Peters *et al*, 2001b] and plasma fibrinogen [Pekkanen *et al*, 2000; Schwartz, 2001], enhanced plasma viscosity [Peters *et al*, 1997] and altered haematological indices [Seaton *et al*, 1999]. In animal studies there are similar reports with increased fibrinogen in the blood of PM-exposed hypertensive rats [Cassee *et al*, 2002] and normal rats exposed to ultrafine carbon particles [Elder *et al*, 2004b]. In a recent clinical study inhalation of carbon ultrafine particles altered leukocyte expression of adhesion molecules in peripheral blood suggesting a systemic inflammatory response [Frampton *et al*, 2006].

1.6.2 Vascular inflammation, atherosclerosis and plaque stability

Atherosclerosis was initially considered a disorder of lipid storage and metabolism, but in recent years evidence has emerged to suggest it is caused by a dynamic inflammatory process of which endothelial dysfunction is one of the earliest features [Ross, 1999]. Endothelial activation results in the upregulation of leukocyte adhesion proteins and initiates local vascular inflammation. The release of growth factors and pro-inflammatory cytokines results in leukocyte and monocyte recruitment, induction of atheroma formation and further arterial damage. Plaque expansion and disruption can lead to angina and acute coronary syndromes, including myocardial infarction [Blum and Miller, 1996; Ross, 1999].

Patients with overt cardiovascular disease [Haverkate *et al*, 1997] and those with cardiovascular risk factors have increased biomarkers of systemic inflammation [Lee and Libby, 1997]. Increased plasma CRP concentrations have been shown to predict the risk of acute myocardial or cerebral infarction independent of other risk factors [Ridker and Haughey, 1998; Ridker *et al*, 2000]. Experimental studies suggest that CRP is not just a biomarker of atherosclerosis, but may have effects on endothelial cell function and may have a direct role in the pathogenesis of atherosclerosis [Verma and Yeh, 2003]. Clinical studies using *Salmonella typhus* vaccination as a model of acute systemic inflammation demonstrate that inflammation can transiently but profoundly alter systemic endothelial function [Hingorani *et al*, 2000]. Indeed, patients with chronic systemic inflammation, such as rheumatoid arthritis, have evidence of significant endothelial dysfunction [Bergholm *et al*, 2002] and accelerated atherosclerosis [Roman *et al*, 2006].

Systemic inflammation follows short-term exposure to increased levels of PM₁₀, with changes in white blood cell and platelet numbers [Ruckerl *et al*, 2007b], interleukin-6 (IL-6) [Ruckerl *et al*, 2007a], CRP [Pope, 2001; Ruckerl *et al*, 2006] and fibrinogen concentrations [Pekkanen *et al*, 2000; Schwartz, 2001], enhanced plasma viscosity [Peters *et al*, 1997] and altered in coagulation Factor VII concentration [Seaton *et al*, 1999]. Experimental exposures confirm these clinical findings [van Eeden *et al*, 2001] and demonstrate direct evidence of systemic inflammation [Salvi *et al*, 1999] and endothelial dysfunction [Vincent *et al*, 2001; Sun *et al*, 2005a].

It is plausible that repeated exposure to PM might induce the vascular inflammation of atherosclerosis and promote plaque expansion or rupture. The first direct evidence to support this hypothesis was provided by Suwa and co-workers [Suwa *et al*, 2002] who demonstrated that instillation of high doses of PM₁₀ resulted in plaque progression and destabilization in an animal model of atherosclerosis. In the same studies PM₁₀ exposure accelerated monocyte release from the bone marrow [Goto *et al*, 2004]. The amount of particulate phagocytosed by alveolar macrophage correlated with both the bone marrow response and plaque volume [Goto *et al*, 2004] suggesting a role for pulmonary inflammation and systemic mediators in determining the proatherogenic effects of PM. In Apolipoprotein E knock out mice, inhalation exposure to ultrafine PM for 6 months increased atherosclerotic plaque volume, altered plaque composition and upregulated vascular inflammation [Chen and Nadziejko, 2005; Sun *et al*, 2005a]. These changes in plaque morphology were

accompanied by abnormal vascular function characterised by exaggerated vasoconstriction and impaired endothelial dependent vasodilatation [Sun *et al*, 2005].

A recent panel study in Los Angeles provided the first evidence of a link between chronic PM exposure and atherosclerosis in man [Kunzli *et al*, 2005]. A 10 $\mu\text{g}/\text{m}^3$ increase in $\text{PM}_{2.5}$ was associated with an increase in carotid intima-media thickness, an ultrasonic measure of atheroma, suggesting that long-term ambient PM exposure may affect the development of atherosclerosis in man. These data together suggest that PM-induced pulmonary inflammation is capable of systemic effects, which can contribute to the progression of atherosclerosis.

1.6.3 Endothelial vasomotor function and endogenous fibrinolysis

In health the vascular endothelium delicately balances regulatory pathways controlling blood flow, coagulation, fibrinolysis and inflammation. It is widely recognized that a variety of risk factors including cigarette smoking can influence vascular tone through endothelium-dependent actions, and there is now extensive evidence of abnormal endothelium-dependent vasomotion in patients with atherosclerosis [Celermajer *et al*, 1996; Ludmer *et al*, 1986; Newby *et al*, 1999]. As combustion products and particulate matter are common to both air pollution and cigarette smoke, we hypothesised that air pollution is likely to have similar detrimental effects on vascular function.

Whilst nitric oxide (NO) dependent vasomotion is important, it may not be representative of other aspects of endothelial function, such as the regulation of

fibrinolysis. Small areas of endothelial denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are usually sub-clinical. Endogenous fibrinolysis and 'passification' of the lesion may therefore be able to prevent thrombus propagation and vessel occlusion [Davies, 2000]. However, in the presence of an adverse pro-inflammatory state or an imbalance in the fibrinolytic system, such microthrombi may propagate, ultimately leading to arterial occlusion and tissue infarction [Rosenberg and Aird, 1999]. Thus, the initiation, modification and resolution of unstable and inflamed atheromatous plaques may be critically dependent on the cellular activation and function of the surrounding endothelium and vascular wall.

The fibrinolytic factor tissue plasminogen activator (t-PA) regulates the degradation of intravascular fibrin and is released from the endothelium through the translocation of a dynamic intracellular storage pool [van den Eijnden-Schrauwen *et al*, 1995]. If endogenous fibrinolysis is to be effective, then the rapid mobilisation of t-PA from the endothelium is essential because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation [Fox *et al*, 1985]. The efficacy of plasminogen activation and fibrin degradation is further determined by the relative balance between the acute local release of t-PA and its subsequent inhibition through formation of complexes with plasminogen activator inhibitor type 1 (PAI-1). This dynamic aspect of endothelial function and fibrinolytic balance may be directly relevant to the pathogenesis of atherothrombosis.

1.6.4 Direct effects of translocated particles on the endothelium and platelets

The nanoparticle component of inhaled PM may influence the cardiovascular system through indirect effects mediated by pulmonary inflammation or through the direct action of particles that have become blood-borne. Translocation of inhaled nanoparticles across the alveolar-blood barrier has been demonstrated in animal studies for a range of nanoparticles delivered by inhalation and instillation [Nemmar *et al*, 2002; Nemmar *et al*, 2001; Kreyling *et al*, 2002; Oberdorster *et al*, 2002]. Whether inhaled nanoparticulate can readily access the circulation in humans is controversial and is the subject of intense research debate.

Once circulating, nanoparticles may interact with the vascular endothelium, or have direct effects on atherosclerotic plaques causing local oxidative stress and pro-inflammatory effects similar to those found in the lungs. Increased inflammation could destabilize the coronary plaque, resulting in rupture, thrombosis and an acute coronary syndrome. Certainly, injured arteries can take up blood borne nanoparticles [Guzman *et al*, 1996], a fact exploited by the nanotechnology industry for both diagnostic and therapeutic purposes in cardiovascular medicine. The intra-arterial infusion of carbon black nanoparticles has a detrimental effect on the mouse microcirculation with upregulation of von Willebrand factor expression and enhanced fibrin deposition on the endothelial surface [Khandoga *et al*, 2004]. These prothrombotic effects are in keeping with toxicological evidence from inhalation studies, which suggest particle exposure may promote thrombogenesis [Nemmar *et al*, 2003a; Nemmar *et al*, 2003b].

Direct effects of particles on endothelial cells

Blood-borne particles could interact directly with vascular endothelial cells. The direct effects of PM on the expression of genes related to thrombogenesis and fibrinolysis has been studied in cultured endothelial cells [Gilmour *et al*, 2005]. PM₁₀ caused a direct, dose dependent and sustained impairment in endothelial cell production of t-PA. Impaired acute t-PA release in the event of intravascular thrombus formation would cause an imbalance between pro-fibrinolytic factors and their endogenous inhibitors preventing thrombus dissolution. Additionally, PM₁₀ caused a concomitant up-regulation of endothelial tissue factor expression, a potent stimulus for thrombus formation. Taken together these observations suggest that PM₁₀ promotes thrombogenesis in particle-exposed endothelium via two distinct but related pathways: enhanced endothelial tissue factor expression and impaired t-PA release.

Direct effects of particles on platelets

Platelets are numerous in the bloodstream and are central to the process of intravascular thrombus formation. Few studies have addressed the direct effects of particles on platelets. In the experimental studies of Nemmar [Nemmar *et al*, 2003a; Nemmar *et al*, 2003b] *in vivo* thrombosis is induced by endothelial injury using the Rose-Bengal model. In hamsters treated with intra-tracheal diesel particles, both arterial and venous thrombosis was increased in a dose dependent manner. Platelets sampled from the instilled animals underwent more aggregation *in vitro*, although it is unclear whether enhanced *in vivo* thrombus formation was due to inflammation or the direct effects of the particles on platelet function. However, platelets isolated

from normal hamsters and treated with diesel exhaust particles (DEP) showed increased aggregation, suggesting that direct effects were at least plausible.

In a more comprehensive study [Radomski *et al*, 2005], a range of manufactured nanoparticles including carbon nanotubes, fullerenes, urban PM and carbon nanoparticles were incubated with platelets *in vitro*. Platelet aggregation was assessed and clear differences in the potency of these particles were evident. Fullerenes were without effect, urban dust and multi-walled nanotubes caused modest aggregation, whilst mixed carbon nanoparticles and single walled nanotubes were highly potent activators of platelets upregulating platelet glycoprotein IIb/IIIa receptor expression and enhancing aggregate formation. In a ferric chloride induced rat carotid artery thrombosis model, a similar potency was observed with particles enhancing the rate of thrombus formation. Unfortunately, there was little characterization of these particles that might have allowed features such as surface area or chemistry to be linked to the variation in effect on platelet aggregation or thrombus formation.

1.6.5 Arrhythmogenesis

Whilst arrhythmias are unlikely to account for many of the manifestations of the adverse cardiovascular effects of air pollution, they may be implicated in hospitalisation for cardiovascular disease and the incidence of sudden cardiac death. Most studies to date have addressed this issue by examining effects on heart rate variability due to its association with an increased risk of cardiovascular morbidity

and mortality in both healthy individuals [Tsuji *et al*, 1996] and survivors of myocardial infarction [Kleiger *et al*, 1987].

Liao and colleagues were the first to report an association between PM_{2.5} and heart rate variability in a panel of elderly subjects [Liao *et al*, 1999]. The authors considered their finding somewhat exploratory, but the analysis revealed an inverse correlation between same day PM_{2.5} concentrations and both the high and low frequency power domains. They hypothesize that an effect of PM exposure on the autonomic control of heart rate and rhythm may explain the association between PM and adverse cardiovascular outcomes. Subsequently numerous panel studies have explored this mechanistic hypothesis by studying the associations between levels of different air pollutants and changes in heart rate variability or incidence of cardiac arrhythmia. Whilst controlled exposure studies to ultrafine carbon particles and sulphur dioxide have attempted to bring clarity [Routledge *et al*, 2006], the current literature is inconsistent in the magnitude, type and direction of these changes, making firm conclusions challenging.

Direct evidence that air pollution may be a trigger for arrhythmia has been further assessed in studies of high-risk patients with implanted cardiac defibrillators (ICDs). In a pilot study, estimated community acquired exposures to fine particulate and other traffic-derived air pollutants were associated with an increase in the number of defibrillator interventions amongst 100 patients with ICDs [Peters *et al*, 2000]. However, in a larger more complete analysis with longer follow up, there was no increase in the risk of ventricular arrhythmia unless the analysis was restricted only

to those patients requiring frequent ICD interventions [Dockery *et al*, 2005]. It should also be recognised that acute myocardial ischaemia secondary to an acute coronary syndrome is the most common trigger for life threatening arrhythmias. Overall, the pro-arrhythmic potential of air pollution remains uncertain and has yet to be definitively established.

1.6.6 Summary

Recent experimental studies suggest multiple plausible mechanistic pathways whereby particles may exert adverse effects on the cardiovascular system. Systemic inflammation and endothelial dysfunction are central to this hypothesis. Particles or soluble components may translocate into the bloodstream, resulting in direct effects on atherosclerotic plaque stability, the vascular endothelium, platelet function and thrombosis. Whether these pathways are relevant in man and underpin the association between air pollution and acute cardiovascular events is at present unclear.

1.7 AIMS AND HYPOTHESES

The principle aim of this thesis is to derive a better understanding of the biological mechanisms whereby inhaled air pollutants influence the cardiovascular system. Specifically we aim to determine the effects of exposure to diesel exhaust and CAPs on vascular and fibrinolytic function in man. Furthermore we hope to establish whether patients with coronary heart disease are more susceptible to the effects of air

pollution. We will explore the role of oxidative stress, inflammation and systemic translocation in determining the vascular effects of these exposures.

Appreciation of the effects of air pollution on the cardiovascular system may have implications in the future prevention, treatment and management of patients with coronary heart disease. Identifying the harmful constituents of urban air pollution would allow a more targeted approach in future environmental health policies to reduce the adverse effects of air pollution on our health.

The following hypothesis will be addressed:

1. Exposure to dilute diesel exhaust causes an acute impairment of endothelial vasomotor and fibrinolytic function in healthy volunteers (Chapter 3)
2. Vascular dysfunction following exposure to diesel exhaust is due to oxidative stress and systemic inflammation (Chapter 4)
3. Inhaled radiolabelled nanoparticles can translocate from the lung into the circulation in healthy volunteers (Chapter 5)
4. Patients with coronary heart disease are more susceptible to the adverse vascular effects of diesel exhaust (Chapter 6)
5. Exposure to concentrated ambient fine and ultrafine particles will similarly impair vascular and fibrinolytic function in patients with coronary heart disease and age-matched healthy controls (Chapter 7)

CHAPTER 2

METHODOLOGY – HUMAN EXPOSURES AND ASSESSMENTS OF VASCULAR AND FIBRINOLYTIC FUNCTION

2.1 GENERAL

2.1.1 Subject Recruitment

Healthy Volunteers

Healthy male non-smoking volunteers (aged between 18 and 70 years) were recruited by way of local advertisement or from a volunteer database held at the University of Umeå. An information sheet was sent to suitable volunteers who fulfilled the inclusion/exclusion criteria outlined below. Participating subjects General Practitioners were informed in writing.

Subjects taking regular medication and those with clinical evidence of atherosclerotic vascular disease, arrhythmia, diabetes mellitus, hypertension (systolic blood pressure >150 mmHg), renal or hepatic failure, asthma, significant occupational exposure to air pollution, or an inter-current illness likely to be associated with inflammation were excluded from the study. Subjects had normal lung function and reported no symptoms of respiratory tract infection for at least six weeks prior to or during the study.

Patients with Coronary Heart Disease

Potential subjects were identified from databases of patients who had undergone coronary angiography within the Royal Infirmary of Edinburgh or University Hospital Umeå. A study information sheet was posted to suitable subjects meeting study inclusion/exclusion criteria defined below. The General Practitioner and Consultant Cardiologist responsible for participating subjects care were informed.

All patients had proven coronary heart disease with a previous myocardial infarction (>6 months previously) or had confirmed coronary artery disease on angiography, and were receiving standard secondary preventative therapy. Patients with angina pectoris (Canadian Cardiovascular Society grade ≥ 2), a history of arrhythmia, diabetes mellitus, uncontrolled hypertension, renal or hepatic failure, or those with unstable coronary disease (acute coronary syndrome or unstable symptoms within 3 months) were excluded. All volunteers were invited to a pre-study screening visit for exercise stress testing and patients unable to achieve stage 2 of the Bruce protocol, or patients who had marked ECG changes (left bundle branch block, early ST depression >2mm) or developed hypotension were excluded. Current smokers and those with asthma, significant occupational exposure to air pollution, or an inter-current illness were also excluded from the study.

2.1.2 Ethical Considerations

All studies were undertaken in accordance with the regulations of the Lothian Research Ethics Committee, Umeå Regional Ethical Review Board, and with the Declaration of Helsinki of the World Medical Association. The written informed consent of each subject or patient was obtained before entry into the study. Further details of the ethical review process are available on www.clinicaltrials.gov (reference number NCT00437138).

2.1.3 Subject Preparation

Subjects were requested to abstain from alcohol for 24 hours and food, caffeine-containing drinks and tobacco for at least 4 hours before each study. All medication was continued throughout the study period, with the exception of angiotensin converting enzyme (ACE) inhibitors, which were withdrawn 7 days prior to each vascular study as it augments bradykinin induced endothelial t-PA release [Witherow *et al*, 2002]. All studies were conducted in a quiet temperature-controlled room maintained at 22-25°C.

2.2 EXPOSURES TO DILUTE DIESEL EXHAUST

Controlled exposures to dilute diesel exhaust in healthy volunteers and patients with coronary heart disease were performed using a unique and well-characterised exposure facility in Umeå, Sweden. This exposure facility has been used to study the effects of dilute diesel exhaust on lung inflammation and function in a range of healthy volunteers and patients with pulmonary disease since its inception in 1994. The exposures are standardised to ensure a particle concentration of 300 µg/m³. These concentrations are found on a regular basis in heavy traffic, occupational settings, and in the world's most-polluted cities.

2.2.1 Diesel exhaust exposure and monitoring

The diesel exhaust was generated from an idling Volvo diesel engine (Volvo TD45, 4.5L, 4 cylinders, 680rpm) from Swedish Low Sulphur Gasoil E10 (Preem, Göteborg, Sweden). Over 90% of the exhaust was shunted away, and the remainder diluted with filtered air heated to 20°C (humidity ~50%) before being fed into a whole body exposure chamber (3x3x2.4m) at a steady-state concentration (Figure 2.1).

(a)



(b)



Figure 2.1 (a) Idling diesel engine and (b) whole body exposure chamber for human exposure studies to dilute diesel exhaust.

The chamber was continuously monitored for pollutants with exposures standardised using real time measurements of nitrogen oxides (NO_x) to deliver a particulate matter (PM) concentration of $300 \mu\text{g}/\text{m}^3$. Nitrogen dioxide (NO_2) and nitrogen oxide (NO) were measured online using a chemiluminescence technique (ECO Physics CLD 700 AL Med, Switzerland). A UNOR 610 infrared spectrophotometer (Maihak AG, Hamburg, Germany) was used for online monitoring of carbon monoxide (CO). Total hydrocarbon in the exposure aerosol was measured using a flame ionization detection method (Hydrocarbon Analyzer, Model 3-300, JUM Engineering Co, Oakland, California, USA). Continuous registration of the number of particles $<1 \mu\text{m}$ diameter/ cm^3 was obtained with a condensation particle counter (CPC) (TSI, St Paul, MN, USA). The size distribution of particulate in the exposure chamber was determined by a scanning mobility particle sizer (SMPS) (TSI, Inc., Shoreview, MN, USA).

There was little variation in particle number (1.26 ± 0.01 particles/ cm^3), NO_x (4.45 ± 0.02 parts per million, ppm), NO_2 (1.01 ± 0.01 ppm), NO (3.45 ± 0.03 ppm), CO (2.9 ± 0.1 ppm) and total hydrocarbon (2.8 ± 0.1 ppm) concentrations between exposures. The size distribution of PM in the exposure chamber showed a lognormal distribution, typically ranged from 20 to 120 nm, with a count median diameter of 54 nm (geometric standard deviation = 1.7 nm; Figure 2.2). The polycyclic aromatic hydrocarbons (PAHs) consisted of semi-volatile gaseous compounds with only a minor fraction (3.5%) present as particulate associated material, 0.04% of total PM and 0.06% of the PM organic fraction. The dominating PAHs were phenanthrene,

fluorene, 2-methylfluorene, dibenzothiophene and different methyl-substituted phenanthrenes accounting for approximately 90% of total PAH.

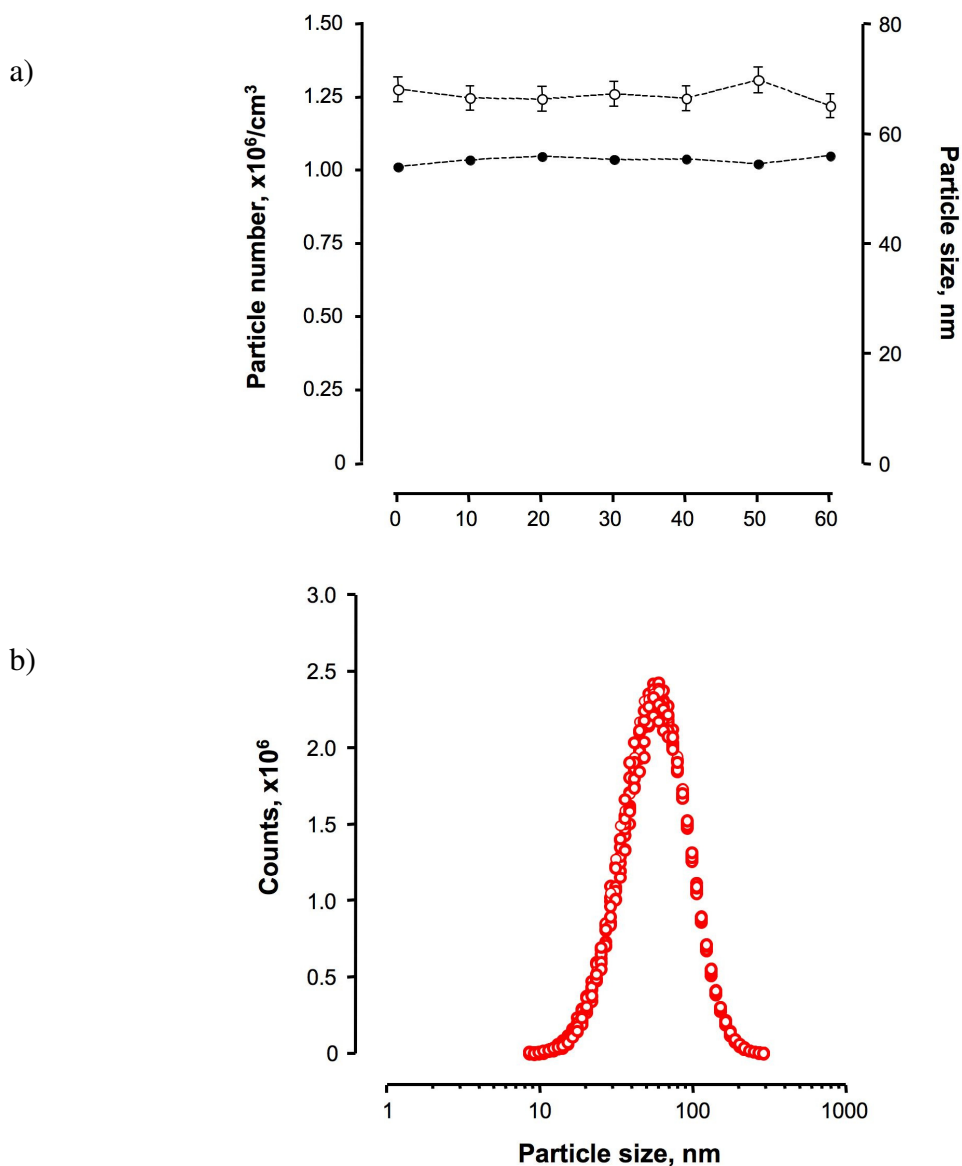


Figure 2.2 a) Particle number (\circ) and count median diameter (\bullet) of particulate during controlled exposure to dilute diesel exhaust for 60 minutes (mean \pm SEM, $n=5$). b) The size of inhaled diesel particulates ($n=5$) showed a lognormal distribution and typically ranged from 20 to 120 nm, with a count median diameter of 54 nm (geometric standard deviation = 1.7 nm).

2.3 EXPOSURES TO CONCENTRATED AMBIENT PARTICLES

An important challenge in this field is to establish the relevant physicochemical properties of the inhaled particles responsible for observed health effects. A major criticism of most mechanistic studies is that the exposure route is artificial and therefore not directly comparable to the epidemiological panel studies. In the last few years, however, instrumentation has been developed that is designed to deliver a continuous flow of air in which the concentration of ambient PM in the sampled air stream is increased in real-time by roughly an order of magnitude [Sioutas *et al*, 1995; Sioutas *et al*, 1997]. The advantage of these instruments is that they can provide genuine inhalation exposures under controlled conditions. In previous studies exposure to fine +ultrafine CAPs has been shown to induce mild pulmonary inflammation in healthy adults [Ghio *et al*, 2000], and to change heart rate variability in the elderly [Devlin *et al*, 2003] and in asthmatic and healthy younger adults [Gong *et al*, 2003].

Our exposures were conducted in collaboration with a group from the Dutch National Institute of the Environment (RIVM). The RIVM have developed a Mobile Ambient Particle Concentrator Exposure Laboratory (MAPCEL) containing a Versatile Aerosol Concentration Enrichment System (VACES) concentrator [Kim *et al*, 2000], with the aim of concentrating fine and ultrafine particles (diameter <2.5 µm) and delivering them to a human exposure chamber at a flow rate of 50 litre per minute. In the first exposure study of its kind, the MAPCEL was transported to

Edinburgh to assess the effects of local CAPs on vascular function in patients with ischemic heart disease and age-matched healthy controls.

The MAPCEL was situated outside the Royal Infirmary Edinburgh approximately 6 miles from the city centre (Figure 2.3). A bus route passed adjacent to the MAPCEL and an arterial city-route was located a few hundred meters away. The primary reason for this choice of location was for safety as this was the first time that an exposure study had been performed in patients with coronary heart disease.

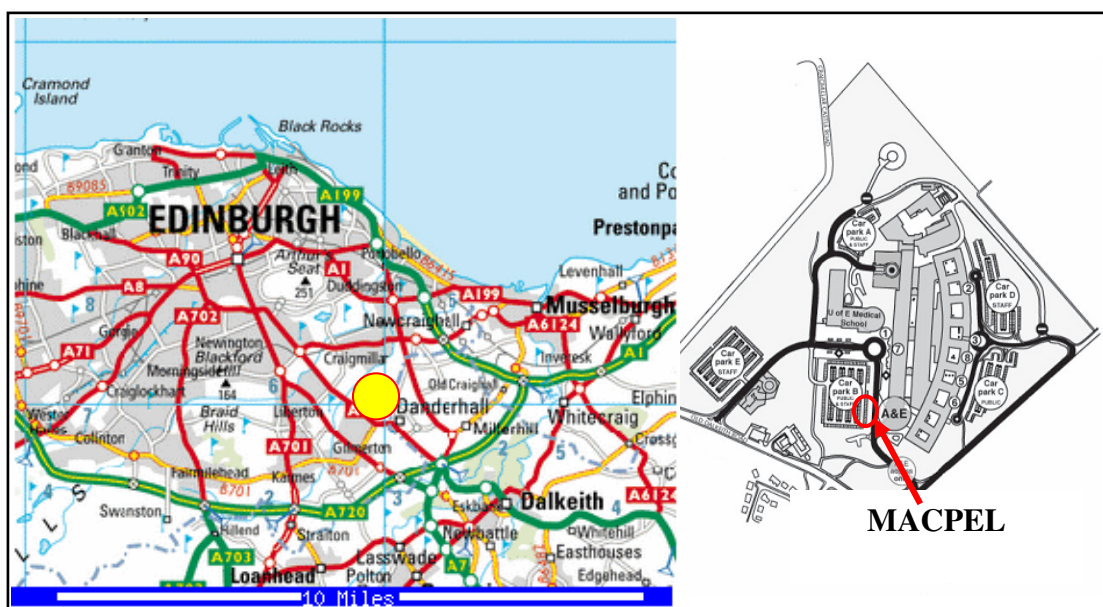


Figure 2.3. Location of the Mobile Ambient Particle Concentrator Exposure Laboratory (MAPCEL) used to conduct human exposures to CAPs.

2.3.1 Enrichment of ambient particulate

A schematic diagram of the MAPCEL and VACES used to deliver CAPs and filtered air to human subjects is given in Figure 2.4. Incoming ambient air (500 L/min) is saturated with water vapour to increase the size of ultrafine and fine particles ($PM_{<2.5}$) before the air is passed through a series of five virtual impactors. This increase in size and therefore mass ensures that particles have sufficient momentum to pass through the impactors exiting in the minor flow (5 L/min) in which the particle concentration is enriched by a factor of 10-20 fold. Of note particles larger than 3 μm are lost by impaction on the walls of the inlet and saturator and do not pass through this system. The outward flow from the five impactors (25 L/min) is desaturated by silica gel dryers to restore the particles to their original size, and diluted with filtered air prior to delivery into the human exposure chamber (50 L/min) resulting in approximately an 8-fold increase in fine + ultrafine particle concentration.

The air in the exposure chamber was continuously monitored for temperature, humidity, nitrogen oxides (chemiluminescence NO-NO₂-NO_x analyser, Thermo Environmental Instruments (TEI), USA), CO (gas filter correlation CO analyser, TEI, USA), sulphur dioxide (SO₂) (pulsed fluorescence SO₂ analyser, TEI, USA), and ozone (photometric analyser, Measurement Controls Corporation, USA). Particle number was determined using a CPC (Model 3022A, Thermo Systems Incorporated, USA). Particle mass was continuously monitored using a DataRam nephelometer (Measuring Instruments for the Environment Corporation, USA) to standardise diesel

exposures, with the precise mass determined by gravimetric filter measurements (Teflon 2.0 μ m 4.7mm, PALL Life Sciences, USA). The concentration of CAPs was not standardised for all subjects as exposures were dependent on ambient particulate levels on the day of the study. We aimed to deliver approximately 200 μ g/m³ to allow comparison with previously published studies [Brook *et al*, 2002].

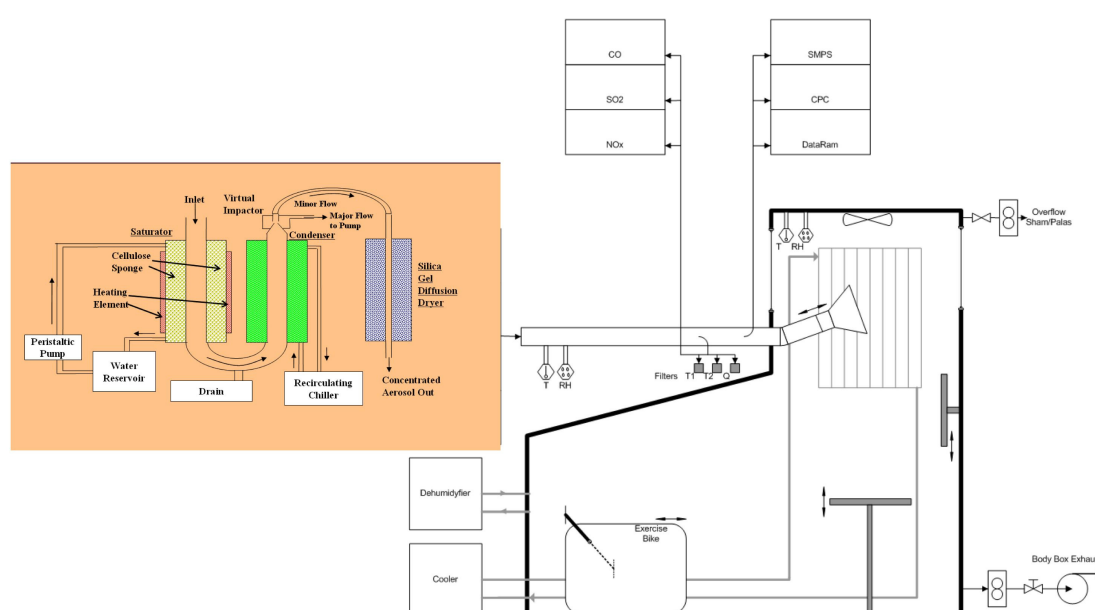


Figure 2.4. A schematic diagram of the VACES used to deliver concentrated fine and ultrafine CAPs and filtered air to human subjects. See text for details.

2.3.2 Assessment of particle size and composition

An Aerosol Time-Of-Flight Mass Spectrometer (ATOFMS) was employed to characterise the particulate matter sampled during CAPs exposures. The operation of ATOFMS instrument has been described in detail elsewhere and only a brief description will be given here [Toner *et al*, 2006]. After entering into the ATOFMS, the particles undergo a jet expansion through a converging nozzle; this results in particle velocities ranging from 100 to 400 m/s. The particles then pass through three stages of differential pumping and form a particle beam. This narrow beam is directed down through the instrument into a light scattering region where the aerodynamic diameter of each individual particle is determined. Particles will scatter light from two continuous wave 532 nm Nd:YAG lasers positioned orthogonal to each other. The signal from the scattered light is sent to a timing circuit that calculates the particles velocity and hence the particles diameter as well as calculating the arrival time for the particle into the ablation region. When the particle arrives in the ablation region, a 266nm Nd:YAG laser is triggered and will ablate the particle and ionise the resulting fragments. The positive and negative ions formed during the laser desorption-ionisation event will be accelerated down the corresponding positive or negative time-of-flight tubes before being recorded by micro-channel plate detectors. These signals are then translated into positive and negative mass spectra for each individual particle sampled.

2.4 EXPOSURES TO TECHNETIUM-LABELLED NANOPARTICLES

Radiolabelled carbon nanoparticles have also been delivered to healthy volunteers via a system designed for diagnostic radionuclide lung ventilation scanning [Nemmar *et al*, 2002]. Although these particles may differ from those produced by the high temperature combustion of fossil fuels, this approach may be a novel way of examining the extent to which nanoparticles are capable of translocating from the lungs into the systemic circulation.

Technegas was generated using a commercially available generator (Technegas generator, Vita Medical Ltd, Sydney) and the manufacturer's recommended protocol. Briefly, 0.14 mL of sodium ^{99m}Tc -pertechnetate ($\text{Na}^{99m}\text{TcO}_4$) solution (2 GBq/mL) was added to a graphite crucible. The solution was evaporated to dryness during the 10-minute simmer phase. The Technegas was then generated during the burn phase in which the crucible was resistively heated at 2550°C for 15 seconds in an atmosphere of 99.998% argon. A size distribution study of Technegas particles using transmission electron microscopy indicated that the majority of particles were 4-20 nm in diameter

2.5 ASSESSMENT OF VASCULAR AND FIBRINOLYTIC FUNCTION

Measuring the response to vasoactive substances released by, or those that interact with, the vascular endothelium in the forearm is a useful measure of endothelial function. Local intra-arterial drug infusion permits the direct assessment of vascular responses without invoking concomitant effects on other organs. In this way the vessels are studied in their physiological environment under the influence of neuronal, circulating, and local mediators [Benjamin *et al*, 1995]. Although measurement of coronary vascular response is of greatest clinical relevance, invasive coronary studies can only really be performed in patients undergoing angiography. The close correlation between coronary and peripheral endothelium-dependent responses [Anderson *et al*, 1995] suggests that endothelial dysfunction may be a systemic state or that circulating factors have parallel effects in both coronary and peripheral arteries [Vita *et al*, 2004].

2.5.1 Venous occlusion plethysmography

This method of assessing resistance vessel function in the forearm is based on the principle of strain gauge venous impedance plethysmography. This technique examines the change in forearm blood flow (FBF) during intra-arterial (brachial artery) administration of agonists at locally active doses (Figure 2.5) [Benjamin *et al*, 1995; Webb, 1995].



Figure 2.5 Intra-brachial artery infusions along with venous occlusion (upper arm) and supra-systolic pressure (wrist) cuffs in a healthy volunteer. A venous cannula for blood sampling is sited in the antecubital vein.

The technique of venous occlusion plethysmography relies on intermittently preventing venous drainage from the arm using upper arm cuffs inflated to above venous pressure whilst arterial inflow is unaltered: blood can enter the forearm but cannot escape. This results in a linear increase in forearm volume over time, which is proportional to arterial blood inflow (Figure 2.6). Under resting conditions, approximately 70% of total forearm blood flow is through skeletal muscle. As the hand contains a high proportion of arteriovenous shunts with a different pharmacology and physiology it is excluded from the circulation by the application of inflation cuffs at supra-systolic pressure during the measurement. The technique of bilateral forearm blood flow measurement is highly reproducible within individuals [Walker *et al*, 2001] and is ideally suited to assessment of interventional strategies with repeated measurements [Wilkinson and Webb, 2001].

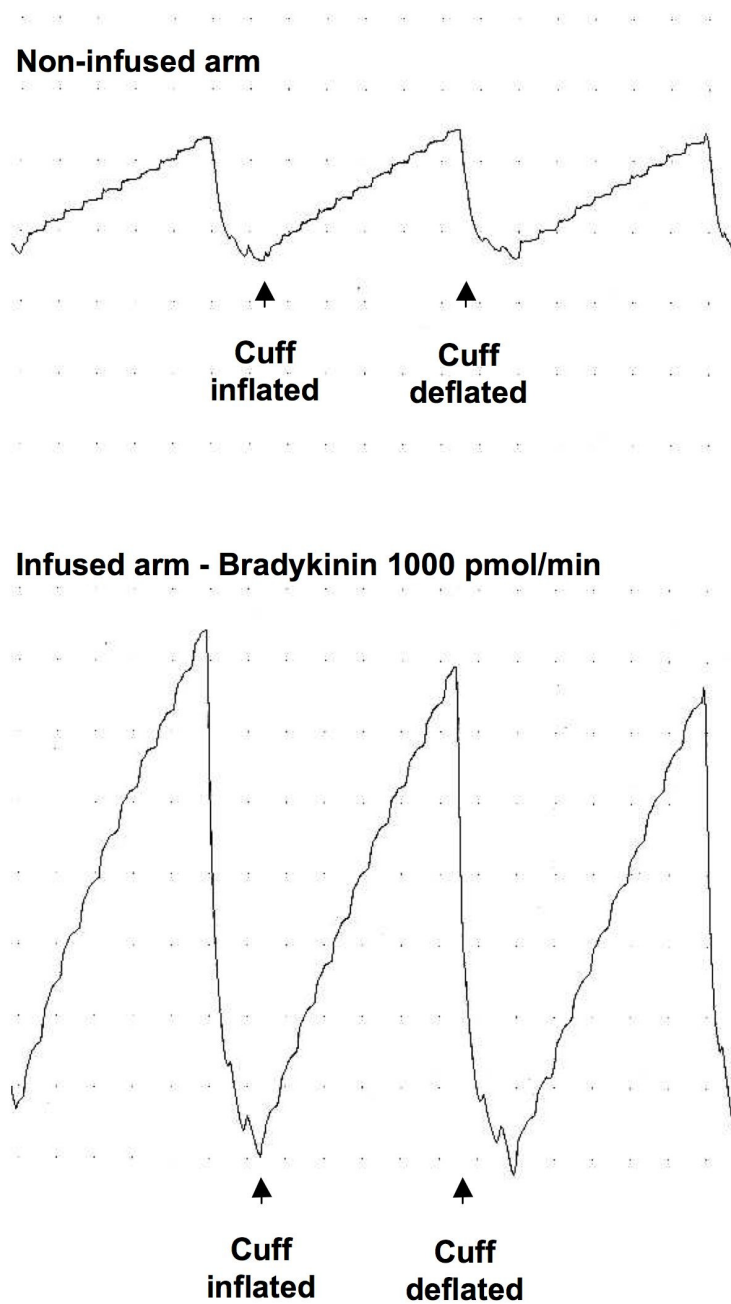


Figure 2.6 Typical blood flow recording from non-infused and infused arms with infusion of intra-brachial bradykinin during venous plethysmography study.

2.5.2 Brachial Artery Cannulation

The brachial artery of the non-dominant arm was cannulated with a 27-G steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-G epidural catheter (Portex Ltd, Hythe, UK) and patency maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via an IVAC P6000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min.

2.5.3 Blood Flow Measurement

Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges that were applied to the widest part of the forearm [Benjamin *et al*, 1995]. Both arms were placed above the level of the right atrium and upper arm cuffs were inflated intermittently to 40 mmHg (venous occlusion) pressure for 10 seconds in every 15 seconds to achieve venous occlusion and obtain plethysmographic recordings. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg (supra-systolic) using E20 Rapid Cuff Inflators (D.E. Hokanson Inc., Washington, USA). Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson Inc.) was processed by a PowerLab® analogue-to-digital converter and Chart™ v5.0.1 software (AD Instruments Ltd, Oxfordshire, UK) and recorded onto a Dell Latitude® laptop (Dell Computers Ltd, UK). Calibration was achieved using the internal standard of the plethysmograph.

2.5.4 Plethysmographic Data Analysis

Plethysmographic data were extracted from the Chart™ data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 2004; Microsoft Corporation, USA). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow that this incurs [Benjamin *et al*, 1995]. Usually, the last five flow recordings in each 3-minute measurement period were calculated and averaged for each arm.

2.5.5 Reproducibility of Plethysmographic Data

A single operator undertook analysis of all data collected during the forearm plethysmography studies. The operator was blinded as to whether blood flow data was obtained following exposure to filtered air or to particulate air pollution. Forearm blood flow responses are reported as absolute blood flow responses (mL/100 mL tissue/min) in the infused and non-infused arm. Previous work has assessed intra-subject variability and determined the coefficient of variation for forearm blood flow across resting conditions and a range of agonists to be between 24-27% [Walker *et al* 2001].

2.6 VENOUS SAMPLING AND LABORATORY ASSAYS

2.6.1 Forearm Venous Sampling

Following administration of local anaesthetic, venous cannulae (17-G) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously [Newby *et al*, 1997]. Blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden; t-PA), trisodium citrate (Monovette®, Sarstedt, Nümbrecht, Germany; PAI-1) potassium ethylene diamine tetra-acetic acid (Monovette®, Sarstedt, Nümbrecht, Germany; cytokines; full blood count) and serum gel (Monovette®, Sarstedt, Nümbrecht, Germany; CRP; clinical chemistry) tubes.

2.6.2 Sample Preparation

Citrate and acidified buffered citrate samples were centrifuged at 2,000 g for 30 minutes at 4°C, EDTA samples at 1000g for 10 minutes at 20°C. Serum samples were centrifuged at 2,000 g for 20 minutes after being allowed to clot on ice. Platelet free plasma or serum was decanted and stored at -80°C before assay.

2.6.3 Fibrinolytic and Inflammatory Assays

Plasma PAI-1 and t-PA antigen concentrations were determined by ELISA: TintElize (Biopool, Trinity Biotech, Wicklow, Ireland) for t-PA and Coaliza (Chromogenix AB, Mölndal, Sweden) for PAI-1. Plasma IL-6, tumor necrosis factor-alpha (TNF- α), soluble P-selectin, and soluble intracellular adhesion molecule-1 (ICAM-1) were measured with commercially available ELISAs (Quantikine, R&D Systems). Intra-

assay, and inter-assay coefficients of variability were 5.2% and 7.2%, and 4.2% and 6.4% for plasma TNF- α and IL-6 respectively. Serum CRP concentrations were determined using with a validated highly sensitive assay (Department of Clinical Biochemistry; Fife NHS Trust, UK) using the method of particle-enhanced immunonephelometry (Behring BN II nephelometer, Dade Behring Inc.). Intra-assay and inter-assay coefficients of variability for hs-CRP were 3.7% and 4.2% respectively. All assays were performed in duplicate and the mean value taken.

Differential white cell count, haematocrit and clinical biochemical assays were undertaken on fresh venous samples in Edinburgh (Departments of Haematology and Clinical Biochemistry, Lothian NHS University Hospitals Trust, UK) and Umeå (Departments of Haematology and Clinical Biochemistry, Umeå Hospital University, Sweden).

2.7 DATA ANALYSIS AND STATISTICS

For the forearm studies, estimated net release of t-PA antigen was defined as the product of the infused forearm plasma flow (based on the haematocrit, Hct and the infused FBF) and the concentration difference between the infused ([t-PA]Inf) and non-infused arms ([t-PA]Non-inf).

Estimated net forearm t-PA release = FBF x (1-Hct) x ([t-PA]Inf - [t-PA]Non-inf)

Area under the curve (AUC) for the dose-response of t-PA release was calculated.

Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures, two-tailed Student's t-test and Chi-squared test using GraphPad Prism (GraphPad Software, California, USA). Results are expressed as mean and standard error of mean (SEM) unless otherwise stated. Statistical significance was assigned at the 5% level.

CHAPTER 3

DIESEL EXHAUST INHALATION CAUSES VASCULAR DYSFUNCTION AND IMPAIRED ENDOGENOUS FIBRINOLYSIS

Published by Mills NL, Törnqvist H, Robinson SD, Darnley K, Gonzales M, Boon NA, MacNee W, Donaldson K, Blomberg A, Sandstrom T, and Newby DE. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. *Circulation* 2005;112:3930-6.

3.1 SUMMARY

Although the mechanisms are unknown, it has been suggested that transient exposure to traffic derived air pollution may be a trigger for acute myocardial infarction. The study aim was to investigate the effects of diesel exhaust inhalation on vascular and endothelial function in man. In a double-blind randomised crossover study, 30 healthy men were exposed to dilute diesel exhaust ($300\text{ }\mu\text{g}/\text{m}^3$ particulate concentration) or air for one hour during intermittent exercise. Bilateral forearm blood flow and inflammatory factors were measured before and during unilateral intra-brachial bradykinin ($100\text{-}1000\text{ pmol}/\text{min}$), acetylcholine ($5\text{-}20\text{ }\mu\text{g}/\text{min}$), sodium nitroprusside ($2\text{-}8\text{ }\mu\text{g}/\text{min}$) and verapamil ($10\text{-}100\text{ }\mu\text{g}/\text{min}$) infusions 2 and 6 hours following exposure. There were no differences in resting forearm blood flow or inflammatory markers following exposure to diesel exhaust or air. Although there was a dose dependent increase in blood flow with each vasodilator ($P<0.0001$ for all), this response was attenuated with bradykinin ($P<0.05$), acetylcholine ($P<0.05$) and sodium nitroprusside ($P<0.001$) infusions two hours following exposure to diesel exhaust and persisted at 6 hours. Bradykinin caused a dose-dependent increase in plasma t-PA ($P<0.0001$) that was suppressed 6 hours following exposure to diesel ($P<0.001$; area under the curve decreased by 34%). At levels encountered in an urban environment, inhalation of dilute diesel exhaust impairs two important and complementary aspects of vascular function in man: the regulation of vascular tone and endogenous fibrinolysis. These important findings provide a potential mechanism that links air pollution to the pathogenesis of atherothrombosis and acute myocardial infarction.

3.2 INTRODUCTION

Air pollution is a major cause of cardiovascular morbidity and mortality. Short-term increases in air pollution exacerbate cardio-respiratory disease leading to hospitalisation for conditions including acute myocardial infarction [Peters *et al*, 2001a]. Long-term repeated exposure increases the risk of cardiovascular mortality with deaths attributable to ischaemic heart disease, arrhythmia, and heart failure [Dockery *et al*, 1993]. These associations are strongest for fine particulate air pollutants [Samet *et al*, 2000], of which the combustion-derived nanoparticulates of diesel exhaust are an important component [Laden *et al*, 2000]. Although significant improvements in air quality have occurred over the last 50 years, the association between PM_{2.5} and mortality is evident below current air quality standards [Ware, 2000].

Despite the strength of the epidemiological evidence and the emergence of promising hypotheses [Brook *et al*, 2004; Pope *et al*, 2004], the important constituents and biological mechanisms responsible for the cardiovascular effects of air pollution are largely unknown. It was recently reported that transient exposure to road traffic may increase the risk of acute myocardial infarction [Peters *et al*, 2004]. Long-term exposure to traffic in those living within 100 meters of a major road, significantly increases cardiopulmonary mortality [Hoek *et al*, 2002]. These important observations suggest that the combustion-derived particulate in PM_{2.5} may be critical in determining the cardiovascular effects of air pollution.

Abnormal endothelial function has been widely recognised in patients with atherosclerosis and its risk factors [Newby *et al*, 2001; Newby *et al*, 1999]. Endothelial dysfunction can also predict the likelihood of future cardiovascular events and death in patients with coronary artery disease [Heitzer *et al*, 2001] and in at risk individuals with normal coronary arteries [Halcox *et al*, 2002]. We have previously demonstrated endothelial dysfunction in both the peripheral and coronary circulation of cigarette smokers [Newby *et al*, 2001; Newby *et al*, 1999]. Given the potential for common etiological factors contained within air pollution and cigarette smoke, we hypothesised that the adverse cardiovascular effects of air pollution are a result of combustion derived particulate and are mediated by an impairment of normal vascular function. Using a carefully characterised exposure system, the study aim was to assess the effect of dilute diesel exhaust inhalation on endothelial vasomotor and fibrinolytic function in man.

3.3 METHODS

3.3.1 Subjects

Thirty healthy male non-smokers between 20 and 38 years old participated in these studies which were performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and the written informed consent of all volunteers. Subjects taking regular medication and those with clinical evidence of atherosclerotic vascular disease, arrhythmia, diabetes mellitus, hypertension, renal or hepatic failure, asthma, significant occupational exposure to air pollution, or an inter-current illness likely to be associated with inflammation were excluded from the study. Subjects had normal lung function and reported no symptoms of respiratory tract infection for at least six weeks prior to or during the study.

3.3.2 Study design

Subjects attended on two occasions two weeks apart and received filtered air or diesel exhaust in a randomised double blind crossover design. Each subject was exposed for one hour in a specially built diesel exposure chamber according to a previously described standard protocol [Rudell *et al*, 1994]. During each exposure they performed moderate exercise (minute ventilation 25 L/min/m²) on a bicycle ergometer that was alternated with rest at 15-minute intervals.

Based on previous exposure [Salvi *et al*, 1999] and systemic inflammatory [Hingorani *et al*, 2000] studies, vascular assessments were performed in 15 subjects at 6-8 hours following diesel or air exposure. In light of our findings from this 6-8 hour study, we subsequently determined vascular function in a further 15 subjects at an earlier time point of 2-4 hours after exposure to diesel exhaust or air. All subjects abstained from alcohol for 24 hours and from food, tobacco and caffeine-containing drinks for at least 4 hours before each vascular study. Studies were carried out in a quiet, temperature controlled room maintained at 22-24°C with subjects lying supine. All subjects remained indoors between the exposure and vascular assessment to minimise additional exposure to particulate air pollution.

3.3.3 Diesel Exposure

The diesel exhaust was generated from an idling Volvo diesel engine (Volvo TD45, 4.5 L, 4 cylinders, 680 rpm) as described previously [Salvi *et al*, 1999]. Over 90% of the exhaust was shunted away, and the remaining part diluted with air and fed into the exposure chamber at a steady-state concentration. The air in the exposure chamber was continuously monitored for NO, NO₂, CO, particles (number/cm³) and total hydrocarbons. The exposures were standardised by keeping the particulate concentration at 300 µg/m³ and were associated with concentrations of NO₂ of 1.6 ppm; NO 4.5 ppm; CO 7.5 ppm; total hydrocarbons 4.3 ppm; formaldehyde 0.26 µg/m³ and 1.2 x 10⁶ suspended particles/cm³. The temperature and humidity in the chamber were controlled at 20°C and 50% respectively.

3.3.4 Vascular studies

All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. Following a 30 min baseline saline infusion, acetylcholine at 5, 10 and 20 µg/min (endothelium-dependent vasodilator that does not release t-PA; Merck Biosciences, Switzerland), bradykinin at 100, 300 and 1000 pmol/min (endothelium-dependent vasodilator that releases t-PA ; Merck Biosciences, Switzerland) and sodium nitroprusside at 2, 4 and 8 µg/min (endothelium-independent vasodilator that does not release t-PA; David Bull Laboratories, UK) were infused for 6 min at each dose. The three vasodilators were separated by 20 min saline infusions and given in a randomised order. In the second cohort with the early (2-4 hour) vascular assessment, verapamil at 10, 30 and 100 µg/min (endothelium and nitric oxide -independent vasodilator that does not release t-PA) was infused at the end of the study protocol [Robinson *et al*, 2006].

Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [Newby *et al*, 1997]. Supine heart rate and blood pressure in the non-infused arm were monitored at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer.

3.3.5 Venous sampling and plasma analysis

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the ante-cubital fossae of both arms. Blood (10 mL) was withdrawn simultaneously from each arm at baseline and during the infusion of each dose of bradykinin, and collected into acidified buffered citrate (Stabilyte tubes, Biopool International) for t-PA assays, and

citrate (BD Vacutainer) for PAI-1 assays. Samples were kept on ice before being centrifuged at 2000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA and PAI-1 antigen concentrations were determined by enzyme linked immunosorbant assays (TintElize t-PA, Biopool EIA; Coaliza PAI-1, Chromogenix AB). Haematocrit was determined by capillary tube centrifugation at baseline and during infusion of bradykinin 1000 pmol/min.

Blood samples were taken immediately before, 2 and 6 hours after the exposure and analysed for total cells, differential count and platelets using an autoanalyzer. Plasma IL-6 and TNF- α were measured using commercially available enzyme-linked immunosorbent assays (Quantikine, R&D Systems, Minneapolis). Plasma immunoreactive big endothelin (ET)-1 and ET-1 concentrations were measured following an acetic acid extraction technique by use of a modified commercial radioimmunoassay with rabbit antihuman big ET-1 or ET-1 (Peninsula Laboratories Europe, St Helens, United Kingdom), as described previously [Adam *et al*, 2001]. Serum CRP concentrations were measured using an immunonephelometric assay (Behring BN II nephelometer, Marburg, Germany).

3.3.6 Data analysis and statistics

Plethysmographic data were analysed as described previously [Newby *et al*, 1999]. Estimated net release of t-PA antigen was defined as the product of the infused forearm plasma flow (based on the mean haematocrit and the infused forearm blood flow) and the concentration difference between the infused and non-infused arms. Continuous variables are reported as mean \pm SEM. Statistical analyses were

performed with GraphPad Prism (Graph Pad Software) using ANOVA with repeated measures and two-tailed Student's *t*-test where appropriate. The area under the curve was calculated for the estimated net release of t-PA during the forearm study period. Statistical significance was taken at $P < 0.05$.

3.4 RESULTS

There were no differences in resting heart rate, blood pressure or baseline forearm blood flow following exposure to diesel exhaust or air in either cohort (Table 3.1).

Table 3.1 Baseline haemodynamic variables

	Air	Diesel	Significance
<i>2 hours, n=15</i>			
Heart rate (bpm)	64 ± 3	65 ± 2	P=0.64
Systolic blood pressure (mmHg)	140 ± 4	148 ± 4	P=0.13
Diastolic blood pressure (mmHg)	71 ± 3	77 ± 4	P=0.08
Infused FBF (mL/100 mL tissue/min)	3.3 ± 0.6	3.1 ± 0.4	P=0.45
Non-infused FBF (mL/100 mL tissue/min)	2.3 ± 0.2	2.6 ± 0.4	P=0.30
<i>6 hours, n=15</i>			
Heart rate (bpm)	61 ± 2	60 ± 2	P=0.66
Systolic blood pressure (mmHg)	138 ± 5	138 ± 3	P=0.39
Diastolic blood pressure (mmHg)	75 ± 2	76 ± 4	P=0.87
Infused FBF (mL/100 mL tissue/min)	3.1 ± 0.5	2.5 ± 0.2	P=0.25
Non-infused FBF (mL/100 mL tissue/min)	2.2 ± 0.1	2.4 ± 0.3	P=0.65

Values are reported as mean ± SEM, two-tailed paired *t*-test

Leucocyte, neutrophil and platelet count, plasma IL-6, TNF- α , Big ET-1 and ET-1, and serum CRP concentrations, were not altered by diesel or air exposure (Table 3.2).

Table 3.2 Systemic effects of exposure to diesel exhaust

	Pre-exposure	2 hours	6 hours
<i>Air</i>			
Leucocytes (x 10 ⁹ cells/L)	5.1 ± 0.2	5.6 ± 0.3	5.3 ± 0.3
Neutrophils (x 10 ⁹ cells/L)	2.8 ± 0.2	3.3 ± 0.2	3.0 ± 0.2
Platelets (x 10 ⁹ cells/L)	217 ± 12	216 ± 9	218 ± 12
IL-6 (pg/mL)	2.6 ± 1.3	-	3.4 ± 0.8
TNF-α (pg/mL)	16.9 ± 1.1	-	17.8 ± 1.2
CRP (mg/L)	0.9 ± 0.3	-	0.8 ± 0.2
PAI-1 antigen (ng/mL)	20.2 ± 3.9	19.2 ± 2.8	18.5 ± 3.7
t-PA antigen (ng/mL)	6.6 ± 0.6	7.0 ± 0.6	6.0 ± 0.5
ET-1 (pg/mL)	4.9 ± 0.5	4.9 ± 0.5	4.9 ± 0.4
Big ET-1 (pg/mL)	28.8 ± 1.5	29.6 ± 2.5	32.2 ± 2.5
<i>Diesel</i>			
Leucocytes (x 10 ⁹ cells/L)	5.6 ± 0.3	5.7 ± 0.4	5.1 ± 0.3
Neutrophils (x 10 ⁹ cells/L)	2.8 ± 0.2	3.4 ± 0.3	2.9 ± 0.3
Platelets (x 10 ⁹ cells/L)	228 ± 14	227 ± 11	221 ± 12
IL-6 (pg/mL)	2.7 ± 0.7	-	4.3 ± 2.2
TNF-α (pg/mL)	15.3 ± 0.5	-	16.0 ± 0.8
CRP (mg/L)	1.0 ± 0.4	-	0.7 ± 0.4
PAI-1 antigen (ng/mL)	16.1 ± 3.0	15.7 ± 3.6	12.8 ± 2.6
t-PA antigen (ng/mL)	6.0 ± 0.6	5.9 ± 0.6	5.3 ± 0.6
ET-1 (pg/mL)	4.5 ± 0.4	4.8 ± 0.3	4.8 ± 0.5
Big ET-1 (pg/mL)	30.2 ± 2.3	31.9 ± 3.7	28.1 ± 2.6

Values are reported as mean ± SEM

Bradykinin, acetylcholine, and sodium nitroprusside caused dose-dependent increases in forearm blood flow following both air and diesel exhaust exposure ($P < 0.0001$; Figure 3.1). The increase in blood flow was blunted 2 hours following exposure to diesel exhaust in response to the infusion of bradykinin ($P < 0.05$), acetylcholine ($P < 0.05$) and sodium nitroprusside ($P < 0.001$) and this diminished response persisted at 6 hours (Figure 3.2). In contrast, verapamil induced vasodilatation was unaffected following exposure to air or diesel exhaust ($P = \text{NS}$).

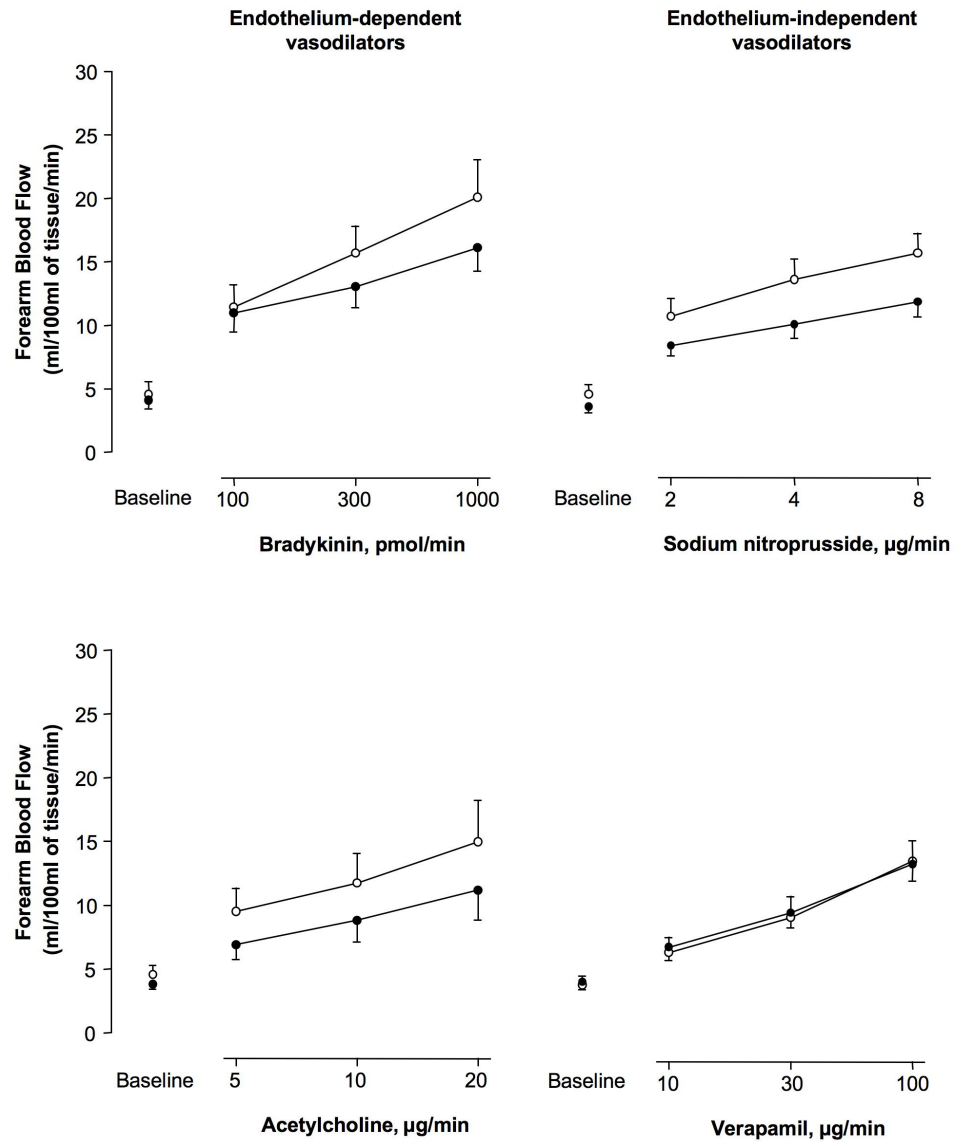


Figure 3.1 Infused forearm blood flow in subjects 2-4 hours following diesel exposure (●) and air (○) during intra-brachial infusion of bradykinin, acetylcholine, sodium nitroprusside and verapamil: for all dose responses $P < 0.0001$. For diesel exposure (●) versus air (○); bradykinin ($P < 0.05$), acetylcholine ($P < 0.05$), sodium nitroprusside ($P < 0.001$), and verapamil ($P = \text{NS}$).

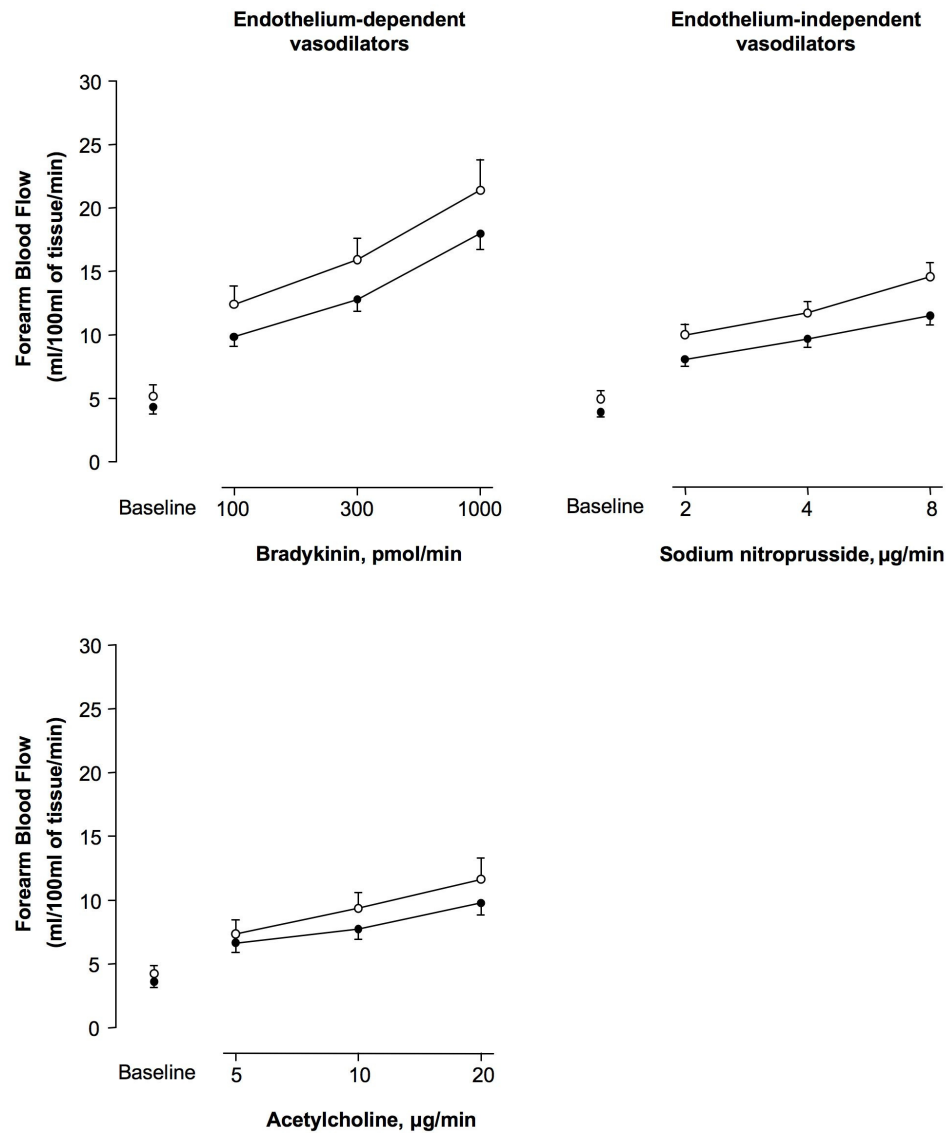


Figure 3.2 Infused forearm blood flow in subjects 6-8 hours following diesel exposure (●) and air (○) during intra-brachial infusion of bradykinin, acetylcholine, and sodium nitroprusside: for all dose responses $P < 0.0001$. For diesel exposure (●) versus air (○); bradykinin ($P < 0.05$), acetylcholine ($P = 0.07$), sodium nitroprusside ($P < 0.001$).

Bradykinin caused a dose-dependent increase in plasma t-PA antigen concentrations ($P<0.0001$; Table 3.3) that was reduced 6 hours following diesel exposure ($P<0.001$). The estimated net t-PA antigen release was reduced by 34% 6 hours following exposure to diesel ($P<0.05$; Figure 3.3), but was unaffected at the earlier time point of 2 hours.

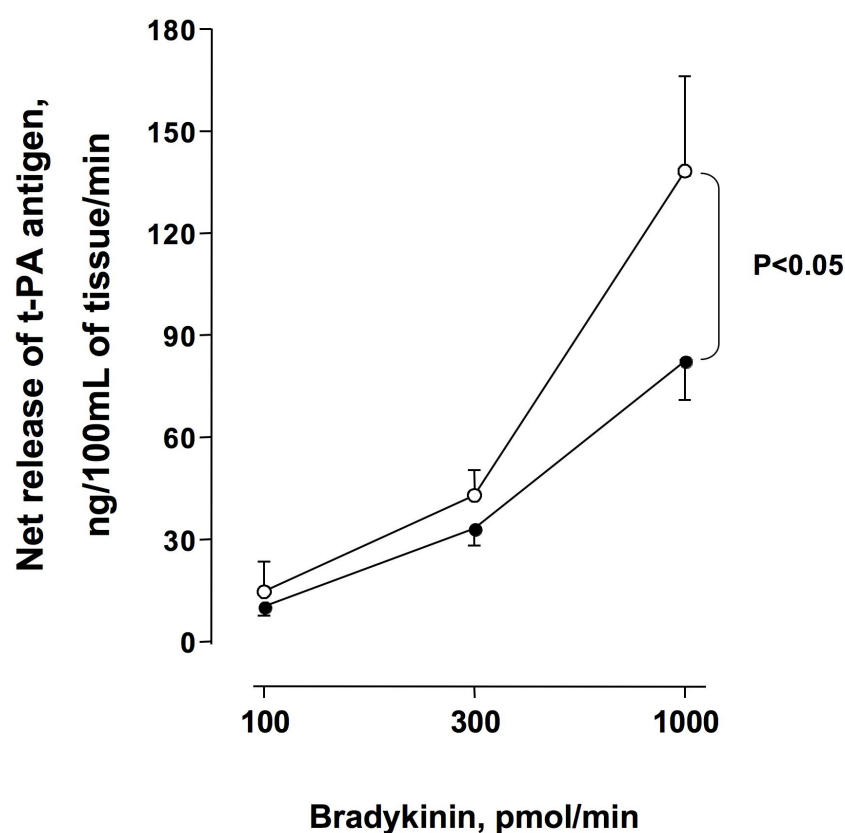


Figure 3.3 Net release of t-PA antigen in subjects 6 hours following diesel exposure (●) and air (○) during intra-brachial infusion of bradykinin: for both dose responses $P<0.0001$. For diesel exposure (●) versus air (○) $P<0.05$.

Table 3.3 Plasma t-PA antigen concentrations following air and diesel exposure

2 hours		Air				Diesel			
Bradykinin, pmol/min	t-PA antigen, ng/mL	0	100	300	1000	0	100	300	1000
Non-infused arm		7.0 ± 0.6	7.0 ± 0.7	7.7 ± 0.8	9.4 ± 1.2	7.2 ± 0.8	7.5 ± 0.8	8.0 ± 0.8	8.9 ± 0.8
Infused arm		6.5 ± 0.5	8.8 ± 1.4	12.2 ± 1.9	17.1 ± 1.3*	6.6 ± 0.7	9.8 ± 1.5	13.7 ± 0.9	18.7 ± 2.7*
Difference		-0.5 ± 0.3	1.7 ± 0.8	4.6 ± 1.2	9.3 ± 1.1*	-0.3 ± 0.4	2.2 ± 0.8	5.3 ± 1.4	9.9 ± 2.2*
Net t-PA release, ng/100ml of tissue/min		-3.3 ± 2.2	6.6 ± 2.7	27.3 ± 4.3	81.3 ± 10.7*	-0.2 ± 1.0	8.9 ± 3.0	29.4 ± 6.1	82.9 ± 17.8*
6 hours		Air				Diesel			
Bradykinin, pmol/min	t-PA antigen, ng/mL	0	100	300	1000	0	100	300	1000
Non-infused arm		5.8 ± 0.5	7.1 ± 1.1	7.0 ± 0.6	7.7 ± 0.6	5.5 ± 0.6	5.6 ± 0.5	5.7 ± 0.4	7.1 ± 0.5
Infused arm		6.0 ± 0.5	9.6 ± 1.7	12.0 ± 1.3	18.6 ± 2.5*	5.3 ± 0.6	7.4 ± 0.9	10.0 ± 1.0	14.5 ± 1.3*‡
Difference		0.2 ± 0.2	2.6 ± 1.7	5.4 ± 1.1	11.7 ± 2.5*	-0.2 ± 0.2	2.0 ± 0.6	4.6 ± 0.8	8.0 ± 1.1*†
Net t-PA release, ng/100ml of tissue/min		0.7 ± 0.9	15.8 ± 9.4	42.9 ± 7.3	138.2 ± 27.8*	-1.0 ± 0.5	10.6 ± 2.9	33.2 ± 5.1	82.5 ± 11.5*†

Values are reported as mean ± SEM; ANOVA (dose response), *P<0.0001; ANOVA (air vs. diesel), †P<0.05, ‡P<0.001.

3.5 DISCUSSION

This is the first study to demonstrate that inhalation of diesel exhaust, a common urban air pollutant, can impair vascular function in man. Using a robust and powerful study design, we have assessed two important and complementary aspects of vascular function: the regulation of vascular tone and endogenous fibrinolysis. Both are impaired and plausibly related to the well-documented cardiovascular effects of air pollution. These important findings provide a plausible mechanism that links air pollution to the pathogenesis of atherothrombosis and acute myocardial infarction.

3.5.1 Vasomotor function

Impaired endothelium-dependent and -independent vasomotor function in the forearm vascular bed is associated with an increased risk of acute cardiovascular events including cardiac death [Heitzer *et al*, 2001]. We demonstrate that inhalation of diesel exhaust impairs vasomotor responses to both endothelium-dependent and -independent vasodilators at 6 hours. On the basis of this initial study, it is not clear whether the impairment is primarily mediated by the vascular endothelium or is a result of smooth muscle dysfunction. However, reduced nitric oxide bioavailability in the presence of increased systemic or vascular oxidative stress is an attractive hypothesis.

The endothelium is a major target of oxidative stress and this interaction plays an important role in the pathophysiology of vascular disease [Lum and Roebuck, 2001]. Superoxide radicals produced as a consequence of oxidative stress, combine with

nitric oxide to form peroxynitrite, reducing nitric oxide bioavailability in the vessel wall and shifting the balance towards vasoconstriction. In vascular smooth muscle cells, superoxide inhibits the activity of enzymes, such as soluble guanylyl cyclase [Mulsch *et al*, 1997] and cGMP-dependent protein kinase [Oelze *et al*, 2000], thereby reducing both endothelium-dependent and –independent nitric oxide mediated vasodilatation.

We hypothesised that our initial findings were due to the oxidative effects of diesel exhaust, and as such, vascular impairment would occur early. In the subsequent study, we demonstrate an acute impairment to endothelium-dependent and -independent vasodilators, but we were also able to show that vasodilation to the calcium channel antagonist verapamil was unaffected. This suggests that the mechanism of vascular dysfunction involves increased consumption of nitric oxide, whether it be endogenously derived from endothelial nitric oxide synthase, or from an exogenous source such as sodium nitroprusside. Indeed, *in vitro* studies provide support for this mechanism, with Ikeda *et al* demonstrating that the incubation of aortic ring preparations with diesel exhaust particles results in a dose dependent inhibition of acetylcholine-mediated relaxation, an effect abolished by co-incubation with superoxide dismutase [Ikeda *et al*, 1995b].

Our findings of an acute effect of exposure to air pollution are consistent with recent epidemiological studies that report a significant increase in risk of acute myocardial infarction as little as two hours after exposure to road traffic [Peters *et al*, 2004] or an increase in PM_{2.5} [Peters *et al*, 2001a]. Our studies add to those of Brook *et al* who

demonstrated a reduction in brachial artery diameter immediately after exposure to a mixture of concentrate ambient particles and ozone [Brook *et al*, 2002]. In contrast, they did not find an effect on endothelium-dependent or -independent vasodilatation using flow mediated and nitro-glycerine induced dilatation. This may reflect differences in the potency of the pollution models used or the technique used to assess vascular function. Exposures to concentrated ambient particulate are inherently variable in magnitude and composition, where as in our study each volunteer received a standard exposure to combustion derived particulate of known toxicity. Alternatively, it is possible that the vascular effects of particulate matter are mediated primarily in the resistance vessels assessed by plethysmography rather than in the conduit arteries assessed by ultrasound of the brachial artery.

3.5.2 Fibrinolytic function

Acute endogenous t-PA release from the endothelium regulates the dissolution of intravascular thrombosis and is a critical determinant of cardiovascular outcome. This is exemplified by the clinical observation that in approximately 30% of patients with acute myocardial infarction, spontaneous reperfusion occurs within 12 hours of vessel occlusion. The increased risk of atherothrombosis and myocardial infarction in cigarette smokers is at least in part explained by impaired fibrinolytic capacity [Newby *et al*, 2001; Newby *et al*, 1999].

We have described an impairment in acute endogenous fibrinolytic capacity following diesel exhaust inhalation. This abnormality may have prothrombotic consequences that could plausibly result in acute cardiovascular events [Peters *et al*,

2004]. Tissue plasminogen activator release was reduced 6 hours following exposure, but not at the earlier time point, suggesting that this impairment is mediated by an inducible pathway or a change in protein synthesis. Indeed, culture of human umbilical vein endothelial cells with particulate matter for 6 hours inhibits both the synthesis and release of t-PA in a dose dependent manner [Gilmour *et al*, 2005]. Given that cigarette smoking and air pollution share common toxicological properties, the present findings are consistent with previous observations in the peripheral [Newby *et al*, 1999] and coronary [Newby *et al*, 2001] circulations of cigarette smokers, and suggest a potential common etiological factor.

3.5.3 Air pollution, oxidative stress and inflammation

A substantial body of evidence supports a role for oxidative stress in determining the toxicity of ambient pollution [Donaldson *et al*, 2003] and in the pro-inflammatory effects of diesel exhaust particles [Baeza-Squiban *et al*, 1999; Nel *et al*, 2001]. Reactive oxidant species arise not only from the redox potential of the pollutants themselves, but also from the activation of alveolar epithelial cells or resident macrophage and the recruitment of circulating neutrophils.

The potential for inhaled nanoparticulate air pollution to cause local inflammation is not in doubt, and airway neutrophilia has been demonstrated in a healthy volunteer study using the same concentration of diesel particulate and exposure system [Salvi *et al*, 1999]. In our study inhaled diesel exhaust was not associated with an increase in blood leukocytes, plasma IL-6 and TNF- α , or serum CRP concentrations, but this

does not rule out the influence of other circulating inflammatory factors, oxidised lipids or proteins.

3.5.4 Population risk and exposure

As an important source of combustion-derived particulate, diesel exhaust is strongly implicated in the observed adverse effects of air pollution [Brunekreef and Holgate, 2002; Laden *et al*, 2000; Pope *et al*, 1999]. Particulate matter concentrations can regularly reach levels of $300 \mu\text{g}/\text{m}^3$ in heavy traffic, occupational settings, and in the world's largest cities [Report, 1994]. Exposure to $300 \mu\text{g}/\text{m}^3$ for one hour increases a person's average exposure over a 24 hour period by only $12 \mu\text{g}/\text{m}^3$. Changes of this magnitude occur on a daily basis in even the least polluted of cities, and are associated with increases in cardiorespiratory mortality [Samet *et al*, 2000]. Our model is therefore relevant both in the composition and magnitude of exposure for the assessment of short-term health effects in man.

Diesel exhaust is a complex mixture of gases and particles, and from our findings we cannot exclude a non-particulate cause of the adverse vascular effects. However, in epidemiological studies, particulate matter has been held responsible for the majority of the adverse health effects of air pollution [Schwartz, 1994]. Ambient nitrogen dioxide can be considered a surrogate for traffic derived pollution [Brunekreef and Holgate, 2002], but has little adverse effect in controlled chamber studies even at the exposure levels seen here [Ayres, 1998]. There are no reports of the potential adverse cardiovascular effects of toxins such as hydrocarbons or formaldehyde. We therefore suggest that the vascular effects described above are mediated primarily by diesel

exhaust particulate and not its other components, but this needs to be more definitively addressed.

3.5.5 Conclusions

Exposure to increased levels of combustion derived air pollution for as little as one hour can impair vasomotor function and endogenous fibrinolysis in man. We provide evidence that this may be the result of reduced nitric oxide bioavailability in the vasculature, and postulate that this effect is mediated by oxidative stress induced by the nanoparticulate fraction of diesel exhaust. These data provide a plausible mechanistic link to explain the association between air pollution and acute myocardial infarction.

CHAPTER 4

PERSISTENT ENDOTHELIAL DYSFUNCTION FOLLOWING DIESEL EXHAUST INHALATION IN MAN

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in man. *Am J Respir Crit Care Med* 2007;176:395-400.

4.1 SUMMARY

Exposure to combustion-derived particulate air pollution is associated with an early (1-2 hours) and sustained (24 hour) rise in cardiovascular morbidity and mortality. We have previously demonstrated that inhalation of diesel exhaust particulate causes an immediate (within 2 hours) impairment of vascular and endothelial function in man. We investigate the vascular and systemic effects of diesel exhaust 24 hours following inhalation in man. Fifteen healthy men were exposed to diesel exhaust (particulate concentration, $300 \mu\text{g}/\text{m}^3$) or filtered air for one hour in a double-blind randomised crossover study. Twenty-four hours following exposure, bilateral forearm blood flow, and inflammatory and fibrinolytic markers were measured before and during unilateral intra-brachial bradykinin, acetylcholine, sodium nitroprusside and verapamil infusions. Resting forearm blood flow, blood pressure and basal fibrinolytic markers were similar 24 hours following either exposure. Diesel exhaust increased plasma cytokine concentrations (tumour necrosis factor- α and interleukin-6; $P < 0.05$ for both) but appeared to reduce acetylcholine ($P = 0.01$) and bradykinin ($P = 0.08$) induced forearm vasodilatation. In contrast, there were no differences in either endothelium-independent (sodium nitroprusside and verapamil) vasodilatation or bradykinin-induced acute plasma t-PA release. Twenty-four hours after diesel exposure, there is a selective and persistent impairment of endothelium-dependent vasodilatation that occurs in the presence of mild systemic inflammation. These findings suggest that combustion-derived particulate air pollution may have important systemic and adverse vascular effects for at least 24 hours following exposure.

4.2 INTRODUCTION

The link between ambient PM air pollution and increased cardio-respiratory mortality and morbidity is well established [Brunekreef and Holgate, 2002]. Short-term exposure to traffic and ambient air pollution is associated with an increased risk of early (1-2 h) and delayed (24 h) presentation with acute myocardial infarction, [Peters *et al*, 2001a; Peters *et al*, 2004] or rehospitalisation for myocardial ischemia in patients with prior myocardial infarction [von Klot *et al*, 2005]. Long-term repeated exposure to PM pollution increases the risk of cardiovascular mortality with deaths attributable to ischemic heart disease, arrhythmia, heart failure and cardiac arrest [Brook *et al*, 2004; Hoek *et al*, 2002; Pope *et al*, 2004] These associations are strongest for fine particulate air pollutants (≤ 2.5 μm in diameter; PM_{2.5}) [Laden *et al*, 2000]. Diesel exhaust emissions are a significant source of PM_{2.5} in urban environments, particularly in Europe where the use of diesel engines in transport has increased steadily in recent years [Charron and Harrison, 2005]. As a consequence, diesel exhaust exposures have been used as models of PM pollution in experimental studies [Rudell *et al*, 1994; Salvi *et al*, 1999; Stenfors *et al*, 2004].

The biological mechanisms underlying the cardiovascular effects of particulate matter air pollution are largely unknown, although it has been suggested that pulmonary inflammation results in systemic consequences that adversely affect the cardiovascular system [Seaton *et al*, 1995]. *In vitro* studies, animal models and human exposures have clearly established the oxidant and pro-inflammatory nature of combustion-derived particulate matter and implied a role for oxidative stress in

determining the toxicity of ambient air pollution and the pro-inflammatory effects of diesel exhaust particulates [Donaldson *et al*, 2005a; Donaldson *et al*, 2005b; Nel *et al*, 2001]. At levels encountered in an urban environment, we have previously demonstrated that exposure to diesel exhaust causes a pronounced airways inflammation including recruitment of inflammatory cells, the upregulation of vascular endothelial adhesion molecules and the enhanced epithelial expression of cytokines. These effects are associated with the upregulation of important oxidative stress-related transcription factors and MAP kinases in the bronchial epithelium [Behndig *et al*, 2006; Pourazar *et al*, 2004; Pourazar *et al*, 2005; Salvi *et al*, 2000].

Endothelial dysfunction is widely considered to represent the earliest pathologic process in atherosclerosis, [Weissberg, 1999] with established risk factors for cardiovascular disease adversely effecting endothelial function [Newby *et al*, 2001; Newby *et al*, 1999]. In recent studies, we have demonstrated an immediate impairment of vascular and endogenous fibrinolytic function in young healthy volunteers following exposure to diesel exhaust [Mills *et al*, 2005]. In the absence of systemic inflammation up to six hours after exposure, we suggested that these early vascular effects were determined by oxidative stress. The duration of these adverse vascular effects are unknown, and the potential for developing later pulmonary and systemic inflammatory effects to potentiate vascular dysfunction require further investigation. The aim of the present study was to investigate whether there is systemic inflammation and sustained vascular dysfunction in healthy volunteers 24 hours after exposure to diesel exhaust.

4.3 METHODS

4.3.1 Subjects

Fifteen healthy, male non-smokers (mean age 26 years: range 18 to 38) participated in the study. Subjects taking regular medication and those with clinical evidence of atherosclerotic vascular disease, arrhythmias, diabetes mellitus, hypertension, renal or hepatic failure, asthma, significant exposure to occupational air pollution or intercurrent illnesses associated with inflammatory diseases were excluded from the study. All subjects had normal lung function and reported no symptoms to suggest respiratory tract infection for at least six weeks prior to and throughout the duration of the study.

4.3.2 Study design

Subjects were exposed to filtered ambient air and diesel exhaust at a particulate matter concentration of $300 \mu\text{g}/\text{m}^3$ for one hour, in a randomised double-blind cross-over fashion, according to a previously described standard protocol [Rudell *et al*, 1994]. The two exposures were performed at least two weeks apart. During each exposure, subjects alternated moderate exercise on a bicycle ergometer and rest at 15-minute intervals. The workload required to generate a ventilation rate of $25 \text{ L}/\text{min}/\text{m}^2$ body surface was determined for each individual. This ensured that all subjects inhaled approximately the same volume of diesel exhaust air pollution and that each subject undertook the same amount of physical activity during both filtered air and diesel exhaust exposures. Systemic inflammatory markers were collected and vascular assessments were performed 24 hours following both exposures. The

subjects were asked not to consume alcohol or caffeine containing drinks for 24 hours and were fasted for at least 4 hours before the vascular assessment. To minimize the risk of confounding by exposure to ambient air pollution, the subjects remained indoors between the exposure and vascular study.

4.3.3 Diesel Exposure

The diesel exhaust air pollution was generated from an idling Volvo diesel (Volvo TD45, 4.5 L, 4 cylinders, 680 rpm, Volvo, Sweden) engine as previously described [Salvi *et al*, 1999]. To reach a steady state concentration, more than 90 % of the diesel exhaust was shunted away and the remaining part diluted with air and fed into the exposure chamber. The air in the exposure chamber was continuously monitored for the gaseous components NO_x, NO, NO₂, and total hydrocarbons. Temperature and humidity were set at 20°C and 50% respectively. The exposures were standardised using continuous monitoring of NO_x ensuring a particulate concentration of approximately 300 µg/m³. Levels of gaseous pollutants were consistent between exposures with a NO_x concentration of 4.44±0.02 ppm, NO₂ concentration of 0.82±0.01 ppm, NO of 3.62±0.02 ppm, and total hydrocarbons of 2.21 ± 0.12 ppm.

4.3.4 Vascular studies

The vascular studies were carried out in a quiet room with the temperature maintained at 22-24°C and with the subject in the supine position. Brachial artery cannulation with a 27-standard wire gauge steel needle was performed under controlled conditions and followed by a 30-min baseline saline infusion before

acetylcholine was infused at 5, 10 and 20 µg/min (endothelium-dependent vasodilator that does not release t-PA; Novartis Pharmaceuticals UK Ltd, Frimley Surrey, UK), bradykinin at 100, 300 and 1,000 pmol/min (endothelium-dependent vasodilator that releases t-PA; Merck-Biosciences, Läfelfingen, Switzerland) and sodium nitroprusside at 2, 4 and 8 µg/min (endothelium-independent vasodilator that does not release t-PA; Hospira INC, Lake Forest, Illinois, USA). The three vasodilators were infused for 6 min at each dose in a randomised order and separated by 20 min saline infusions. After another 20 min saline infusion, verapamil was infused at 10, 30 and 100 µg/min (endothelium- and nitric oxide-independent vasodilator that does not release t-PA; Abbott, Solna, Sweden) at the end of the study protocol.

Forearm blood flow was measured in both arms by venous occlusion plethysmography with a mercury-in-silicone elastomer strain gauges as described previously [Newby *et al*, 1999]. Supine heart rate and blood pressure in the non-infused arm were monitored at intervals in each study with a semi-automated, non-invasive, oscillometric sphygmomanometer (Medicus, Bosch + Sohn, Jungingen, Germany).

4.3.5 Assays

Venous cannulae (17-gauge) were inserted into subcutaneous antecubital veins in both arms. Ten mL of venous blood was withdrawn simultaneously from each arm at baseline and during the infusion of each dose of bradykinin, and collected in acidified buffered citrate (Stabilyte tubes, Trinity Biotech Plc, Wicklow, Ireland) for

t-PA antigen and into citrate (BD Vacutainer, Plymouth, UK) for PAI-1 antigen assays. Plasma samples were kept on ice until centrifuged at 2,000 g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma PAI-1 and t-PA antigen concentrations were determined by ELISA: TintElize (Biopool, Trinity Biotech, Wicklow, Ireland) for t-PA and Coaliza (Chromogenix AB, Mölndal, Sweden) for PAI-1. Haematocrit was determined at baseline and during infusion of bradykinin at 1,000 pmol/min.

Blood samples were drawn from the venous cannulae prior to the onset of the vascular assessment for preparation of serum and plasma approximately 24-hours following each exposure. Plasma IL-6, TNF- α , soluble P-selectin, and soluble ICAM-1 were measured with commercially available ELISAs (Quantikine, R&D Systems).

Plasma nitrite was measured using high performance liquid chromatography based on the methods by Misko TP *et al.* [Misko *et al.*, 1993]. Total anti-oxidant capacity of plasma was measured in Trolox-equivalents as previously described [Miller *et al.*, 1993]. Serum CRP concentrations were measured with an immunonephelometric assay (Behring BN II nephelometer). Whole blood samples were analysed for total cells, differential count and platelets by an autoanalyzer according to standard clinical methods.

4.3.6 Electron paramagnetic resonance (EPR) of diesel particulates

EPR was used to establish radical generation from diesel particulate. Diesel particulate (300µg) was collected on Teflon filters (PTFE Membrane Disc Filter, Pall Corporation, Pall Norden AB, Lund, Sweden) during clinical exposures. To remove particulate matter, filters were vortexed in 2 mL detergent (0.5% Tween20) and particle aggregates broken down by sonication (30 min). 100 µl suspension (particle concentration = 150µg/mL) was withdrawn and incubated with the spin-trap, Tempone-H (1 mmol/L) immediately before the initial measurement. A blank (unexposed) filter was treated in the same way to act as a control. Pyrogallol (100 µmol/L) was used as positive controls to generate superoxide radicals. In some samples superoxide dismutase (SOD; 500 U/mL) was added to scavenge superoxide generated in solution.

Samples were kept at 37°C throughout and measurements were taken at t=1, 20, 40 and 60 min by drawing 50 µl of sample into a capillary tube (Scientific Laboratory Ltd, Coatbridge, UK) and sealing with a plug of soft sealant (Cristaseal, VWR International, Lutterworth, UK). An X-band EPR machine (Magnettech MS-200, Berlin, Germany) was used with the following parameters: microwave frequency, 9.3-9.55 Hz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1500 mG; center field, 3365 G; sweep width, 50 G; sweep time, 30 s; number of passes, 1; receiver gain, 1×10^1 . The intensity scale on all graphs is an arbitrary scale base upon the area under the curve of the first derivative traces generated.

All agents were made freshly before experiment and dissolved in Hank's balanced salt solution (Sigma, Poole, UK) except stock solutions of diesel suspension (dissolved in 0.5% Tween 20) and Tempone-H (dissolved in 0.01 mol/L EDTA). All compounds were purchased from Sigma-Aldrich (Poole, UK) with the exception of Tempone-H (Alexis/Axxora, Nottingham, UK).

4.3.7 Data analysis and statistics

Plethysmographic data were extracted from the Chart data files, and forearm blood flows were calculated for individual venous occlusion cuff inflations as described previously [Newby *et al*, 1999]. Recordings from the first 60 seconds after wrist cuff inflation were not used because of the reflex vasoconstriction this causes. The last 5 flow recordings in each 3-minute measurement period were calculated and averaged for each arm. Estimated net release of t-PA antigen was defined as the product of the infused forearm plasma flow (based on the mean haematocrit and the infused forearm blood flow) and the concentration difference between the infused and non-infused arms.

This study's population size, on the basis of power calculations derived from previous studies, gives 90% power of detecting a 20% difference in t-PA release at a significance level of 5%. Continuous variables are reported as mean \pm SEM. Statistical analyses were performed with GraphPad Prism (Graph Pad Software) using ANOVA with repeated measures when comparing dose response to vasodilators between exposures, and paired two-tailed Student's *t*-test for

comparisons between systemic inflammatory and oxidative stress markers. Statistical significance was taken at $P < 0.05$.

4.4 RESULTS

There were no differences in resting heart rate, blood pressure or baseline forearm blood flow between or during the two study visits (Table 4.1).

Table 4.1 Haemodynamic variables 24-hours following exposure

	Air	Diesel	Significance
Heart rate (bpm)	62 ± 2	61 ± 2	P=0.73
Systolic blood pressure (mmHg)	144 ± 3	143 ± 3	P=0.63
Diastolic blood pressure (mmHg)	71 ± 2	75 ± 2	P=0.16
Infused FBF (mL/100 mL tissue/min)	3.6 ± 0.5	3.2 ± 0.4	P=0.15
Non-infused FBF (mL/100 mL tissue/min)	2.5 ± 0.2	2.7 ± 0.2	P=0.48

Values are reported as mean ± SEM, two-tailed paired *t*-test

4.4.1 Vascular function

Following both air and diesel exhaust exposures, there were dose-dependent increases in the infused forearm blood flow with all vasodilators ($P < 0.001$ for all). After exposure to diesel exhaust, endothelium-dependent vasodilatation was reduced with acetylcholine ($P = 0.01$) and appeared to be reduced with bradykinin ($P = 0.08$; Figure 4.1). In contrast, there were no effects on endothelium-independent vasodilatation: peak blood flow responses to sodium nitroprusside (14.2 ± 1.2 vs. 12.8 ± 0.8 mL/100 mL/min) and verapamil (14.6 ± 0.9 vs. 13.4 ± 0.9 mL/100 mL/min)

were similar. Venous plasma concentrations of nitrite were not effected by exposure (Table 4.2).

Table 4.2 Systemic effects 24-hrs following exposure to air and diesel exhaust

	Air	Diesel	Significance
Leukocytes (x 10 ⁹ cells/L)	5.2 ± 0.4	5.2 ± 0.3	0.84
Neutrophils (x 10 ⁹ cells/L)	2.8 ± 0.2	2.8 ± 0.2	0.57
IL-6 (pg/mL)	1.5 ± 0.2	2.2 ± 0.2	0.02
TNF-α (pg/mL)	0.88 ± 0.07	0.99 ± 0.07	0.02
CRP (mg/mL)	0.75 ± 0.20	0.70 ± 0.19	0.97
sI-CAM-1 (ng/mL)	171 ± 6	181 ± 8	0.76
Nitrite (nM)	108.1 ± 6.8	117.7 ± 8.1	0.45
TEAC (mM)	6.0 ± 0.2	7.1 ± 0.3	0.003
sP-selectin (ng/mL)	33.7 ± 1.8	36.5 ± 1.4	0.02
Platelets (x 10 ⁹ cells/L)	221 ± 12	219 ± 14	0.57
t-PA antigen (ng/mL)	5.9 ± 0.5	6.2 ± 0.6	0.63
PAI-1 antigen (ng/mL)	16.8 ± 2.1	19.8 ± 3.5	0.45

Values are reported as mean ± SEM; Student's *t*-test (air versus diesel)

TEAC = Trolox equivalent antioxidant capacity;

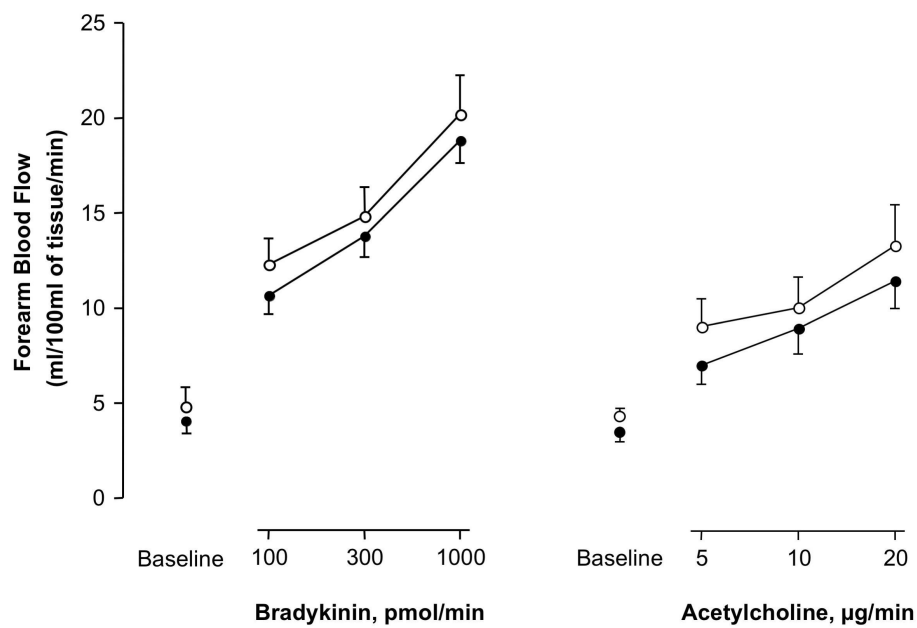


Figure 4.1 Infused forearm blood flow in subjects following diesel exposure (●) and filtered air (○) during intra-brachial infusion of bradykinin and acetylcholine. $P < 0.001$, for all dose responses in the infused arm. For diesel exposure (●) *versus* air (○); bradykinin ($P = 0.08$) and acetylcholine ($P = 0.01$)

Bradykinin caused a dose-dependent increase in plasma t-PA concentrations ($P < 0.001$; Table 4.3) that was unaffected by diesel exhaust exposure. The estimated net t-PA antigen release was similar following exposure to both diesel exhaust and filtered air.

Table 4.3 Forearm blood flow and plasma t-PA antigen concentrations

Air				
Bradykinin, pmol/min	0	100	300	1000
FBF, mL/100 mL/min				
Non-infused arm	2.2 ± 0.2	2.3 ± 0.2	2.2 ± 0.1	2.6 ± 0.3
Infused arm	4.8 ± 1.0	12.3 ± 1.4	14.8 ± 1.5	20.2 ± 2.0*
t-PA antigen, ng/mL				
Non-infused arm	6.2 ± 0.5	6.0 ± 0.5	6.0 ± 0.6	7.8 ± 0.6
Infused arm	6.0 ± 0.5	7.9 ± 0.7	10.1 ± 0.8	14.0 ± 0.9*
Diesel				
Bradykinin, pmol/min	0	100	300	1000
FBF, mL/100 mL/min				
Non-infused arm	2.6 ± 0.3	2.6 ± 0.2	2.5 ± 0.2	2.5 ± 0.3
Infused arm	4.1 ± 0.7	10.7 ± 1.0	13.8 ± 1.1	18.8 ± 1.2*
t-PA antigen, ng/mL				
Non-infused arm	6.0 ± 0.6	6.4 ± 0.5	6.7 ± 0.6	8.4 ± 0.6
Infused arm	6.2 ± 0.6	7.8 ± 0.7	10.6 ± 1.0	14.7 ± 1.3*

Values are reported as mean ± SEM

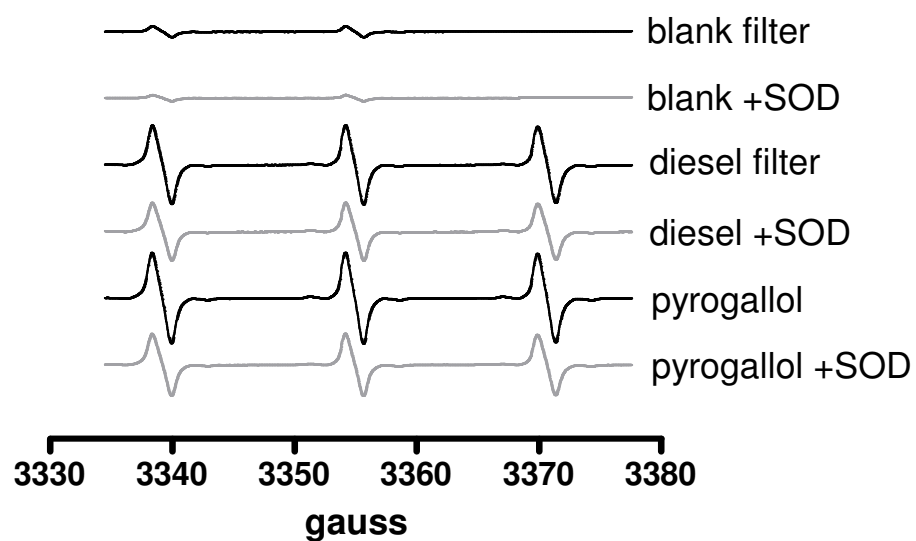
ANOVA (dose response): *P<0.001

4.4.2 Systemic inflammation and oxidative stress

Twenty-four hours following the exposures, there were no differences in leukocyte, and neutrophil counts, or plasma soluble ICAM-1, t-PA and PAI-1 antigen concentrations (Table 4.2). Exposure to dilute diesel exhaust increased plasma IL-6 (2.2 ± 0.2 *versus* 1.5 ± 0.2 pg/mL, $P=0.02$) and TNF- α (0.99 ± 0.07 *versus* 0.88 ± 0.07 pg/mL, $P=0.02$) concentrations compared with air. Total platelet numbers were not affected by exposure, but concentrations of soluble P-selectin were increased 24-hours after exposure to diesel exhaust (36.5 ± 1.4 *versus* 33.7 ± 1.8 ng/mL, $P=0.02$). Leucocyte, neutrophil and platelet count, plasma IL-6, TNF- α , Big ET-1 and ET-1, and serum CRP concentrations, were not altered by diesel or air exposure.

Total anti-oxidant capacity of plasma was greater 24 hours following exposure to diesel exhaust compared to filtered air (Table 4.2). Suspensions of diesel exhaust particles showed a time-dependent increase in the characteristic 3-peak EPR spectrum for a spin-adduct with the unpaired electron in the vicinity of a nitrogen atom, ie. 4-oxo-tempo, the oxidised form of Tempone-H (Figure 4.3a). The signal increased at a constant rate over the 60 min period. EPR measurements were approximately 5-fold higher in suspensions that contained diesel filters (blank, 1144 units; diesel, 5864 units; $t=60$ min; Figure 4.3b). SOD inhibited the EPR signal from diesel, causing a 30.2% reduction in signal. This concentration of SOD had a similar magnitude of effect on the diesel signal as it did to that of the superoxide generator, pyrogallol (34.7% reduction; Figure 4.3b).

a)



b)

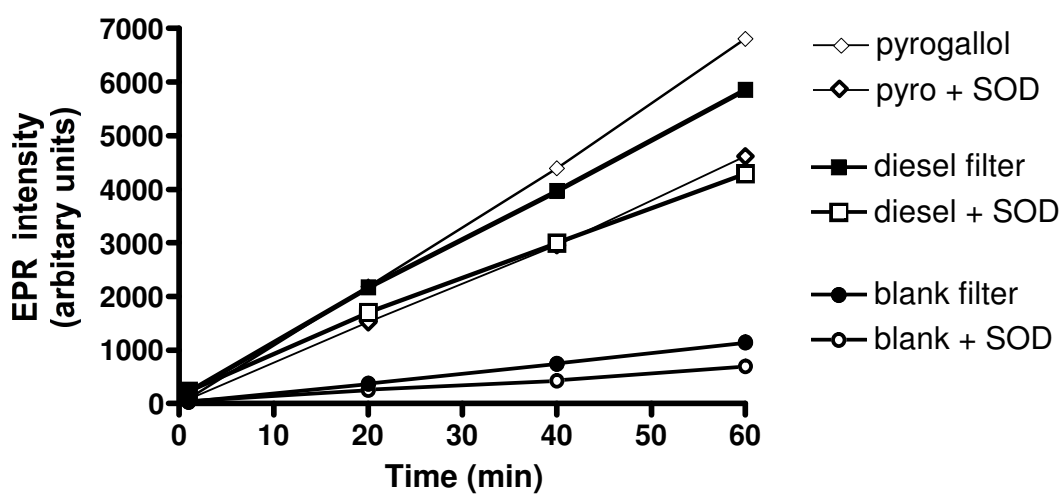


Figure 4.3 (a) Sample EPR spectra generated from suspensions of blank and diesel particulate-coated filters, as well as the superoxide generator pyrogallol. EPR spectra were generated in the presence (grey lines) and absence (black lines) of SOD. (b) Amplitude of EPR spectra (in arbitrary units) over 60 minute incubation period in the presence (open symbols) and absence (closed symbols) of SOD. Diesel particulate

(square symbols) caused a large increase in EPR intensity in comparison to suspension from the blank filter (circles). SOD caused an approximate 30% reduction in the EPR signal from diesel particulate and a similar reduction in signal to the superoxide generator pyrogallol (diamonds).

4.5 DISCUSSION

Twenty-four hours after a transient one-hour exposure to diesel exhaust, there is a selective and persistent impairment of endothelium-dependent vasodilatation that occurs in the presence of mild systemic inflammation following a period of potential oxidative stress. These findings suggest that combustion-derived particulate air pollution may have important adverse systemic and vascular effects for at least 24 hours following exposure. This may account for the epidemiologic observations of a delayed peak in adverse cardiovascular outcomes following exposure to air pollution.

Exposure to air pollution causes airways inflammation and has an important negative effect on respiratory health. Diesel exhaust causes neutrophilic airway inflammation 6 to 24 hours following exposure, [Nordenhall, 2000; Salvi *et al*, 1999; Stenfors *et al*, 2004] increases airways anti-oxidant defences, and activates redox-sensitive transcription factors *in vivo*, consistent with oxidative stress-induced and cytokine-mediated inflammation [Mudway *et al*, 2004]. It is increasingly recognised that these effects may induce important systemic effects [Nemery and Abraham, 2005] including vascular inflammation [Sun *et al*, 2005a].

We hypothesised that our initial observations of an immediate (within 2 hours) impairment of vascular function were due to the oxidative effects of diesel exhaust [Mills *et al*, 2005]. Following exposure to diesel exhaust, endothelium-dependent and -independent nitric oxide donors caused reduced vasodilatation, whilst the endothelium- and nitric oxide-independent vasodilator, verapamil, caused normal

vasodilatation. This pattern of vascular dysfunction suggested increased consumption of nitric oxide, whether it be endogenously derived from endothelial nitric oxide synthase or from an exogenous source such as sodium nitroprusside.

In the current study, we demonstrate a persistent endothelium-dependent vascular dysfunction 24 hours following an hour-long exposure to diesel exhaust. Whilst vasodilatation to both endothelium dependent agonists appeared to be impaired, this only reached statistical significance for acetylcholine. Bradykinin causes vasodilatation through the release of various endothelium-derived factors including nitric oxide, although it is thought that hyperpolarising factor is the primary mediator of this response in man [Honing *et al*, 2000]. It is possible that differences in acetylcholine mediated and bradykinin-mediated vasodilatation may be explained by variation in the relative contribution of nitric oxide to the vasomotor response of these agonists. The mechanism of this selective impairment of endothelium-dependent vasodilatation has not been determined but we suggest that this may be due to modification of endothelial homeostatic pathways following an initial oxidative burst.

The role of vascular oxidative stress in mediating endothelial dysfunction in this clinical model requires confirmation. However, the mechanism is supported by *in vitro* studies, [Ikeda *et al*, 1995a; Ikeda *et al*, 1998; Sun *et al*, 2005b] as well as human exposure studies by our own group [Mills *et al*, 2005] and others [Brook *et al*, 2002]. The endothelium is a major target of oxidative stress and this interaction plays an important role in the pathophysiology of vascular disease [Lum and

Roebuck, 2001]. Incubation of aortic ring preparations with diesel exhaust particles inhibits acetylcholine-mediated relaxation, an effect that can be reversed by co-incubation with the free radical scavenger superoxide dismutase [Ikeda *et al*, 1995b]. Furthermore, diesel exhaust particles can induce oxidative modification of low-density lipoprotein (LDL), the major determinant of atheromatous vascular disease [Ikeda *et al*, 1995a].

Diesel exhaust is a complex mixture of gases and particles, and from our findings we cannot exclude a role for non-particulate or soluble components. Whether diesel particulates or soluble components of the exposure, including organic hydrocarbons and transition metals, can directly affect the systemic vascular endothelium following inhalation also requires clarification. Although evidence that inhaled nanoparticles can translocate into the circulation in man remains controversial, [Mills *et al*, 2006; Nemmar *et al*, 2002] it is not in doubt that diesel exhaust particulates are capable of inducing oxidative stress *in vitro*, with reactive oxidant species arising from the redox potential of the particles themselves and from the activation of inflammatory cells. Using electro-paramagnetic resonance we demonstrate that diesel exhaust particulate is capable of generating oxidative free radicals without prior interaction with pulmonary or vascular tissue. Furthermore, co-incubation of diesel particles with SOD partially prevented this response indicating a contribution of superoxide to this oxidative signal. However, measuring systemic oxidative stress *in vivo* is difficult, as the oxidative state is modulated by a range of anti-oxidant defences [Behndig *et al*, 2006]. Interestingly we demonstrate an increase in the anti-oxidant capacity of plasma 24-hours following exposure to diesel exhaust, perhaps

suggesting upregulation of anti-oxidant defence mechanisms following earlier systemic oxidative stress.

In contrast to our previous study, stimulated release of endothelial t-PA from the forearm circulation was not impaired at 24 hours [Mills *et al*, 2005]. In health, the vascular endothelium delicately balances regulatory pathways controlling coagulation, fibrinolysis and inflammation, as well as regulating vascular tone. It is perhaps not surprising that these complex dynamic functions are altered by exposure to diesel exhaust at different time points. Endogenous fibrinolytic function was impaired at 6-8 hours in our previous studies, but has normalised at 24 hours, suggesting that this aspect of endothelial homeostasis recovers earlier than vasomotor function following exposure to air pollution.

We did not find evidence of a systemic cellular inflammatory response, but did identify changes in pro-inflammatory cytokines IL-6 and TNF-alpha raising the possibility that ongoing airways inflammation is contributing to the state of vascular dysfunction. Observational studies have strongly implicated systemic inflammation as a key pathological mechanism in the health effects of particulate matter [Brook *et al*, 2004]. In panel and population studies, increased PM exposure is associated with an acute phase response with raised serum CRP concentrations, [Peters *et al*, 2001b] increased plasma viscosity [Peters *et al*, 1997] as well as altered haematological indices [Seaton *et al*, 1999] and plasma fibrinogen [Ghio *et al*, 2000; Pekkanen *et al*, 2000]. It is possible that in a susceptible population, in which inflammatory pathways may be upregulated and anti-oxidant defences may be depleted, an hour

long exposure to diesel exhaust would be sufficient to cause a greater systemic inflammatory response. Likewise, repeated exposure over a number of days or weeks may result in inflammation, with prolonged vascular dysfunction contributing to the pathogenesis of atherosclerosis. Indeed, in an ApoE^{-/-} mouse model, long-term exposure to low concentration of PM_{2.5} altered vasomotor tone, induced vascular inflammation and potentiated atherosclerosis [Sun *et al*, 2005a].

Endothelial dysfunction characterised as an impaired vasodilatation to acetylcholine predicts the likelihood of future cardiovascular events and death in patients with coronary artery disease [Heitzer *et al*, 2001] and in at risk individuals with normal coronary arteries [Halcox *et al*, 2002]. Whilst the mechanism of this association has not been precisely identified, this vascular dysfunction clearly has important clinical implications. Our findings of endothelial dysfunction 24 hours following diesel exhaust inhalation suggests that the adverse cardiovascular effects of combustion-derived air pollution are mediated through persistent detrimental vascular effects.

Conclusions

In healthy volunteers, inhalation of dilute diesel exhaust for one hour, at particle concentrations encountered in an urban setting, causes mild systemic inflammation and an impairment of vascular endothelial function that persisted for up to 24 hours after the exposure. This occurred in the absence of alterations in endogenous fibrinolytic capacity. These findings provide a plausible explanation for the observed increase in acute cardiovascular events 24 hours following a peak in traffic-related particulate matter air pollution.

CHAPTER 5

CAN INHALED CARBON NANOPARTICLES TRANSLOCATE INTO THE CIRCULATION IN MAN?

Published by Mills NL, Amin N, Robinson SD, Anand A, Davies J, de la Fuente J,
Boon NA, MacNee W, Millar AM, Donaldson K, and Newby DE. Can inhaled
carbon nanoparticles translocate into the circulation in humans?

Am J Respir Crit Care Med 2006;173:426-31.

5.1 SUMMARY

Increased exposure to particulate air pollution is a risk factor for death and hospitalisation with cardiovascular disease. It has been suggested that the nanoparticulate component of PM₁₀ is capable of translocation into the circulation with the potential for direct effects on the vasculature. The study aim was to determine the extent to which inhaled Technetium-99m (^{99m}Tc) labelled carbon nanoparticles (Technegas), were able to access the systemic circulation. Ten healthy volunteers inhaled Technegas and blood samples were taken sequentially over the following 6 hours. Technegas particles were 4-20 nm in diameter and aggregated to a median particle diameter of approximately 100 nm. Radioactivity was immediately detected in blood, with levels increasing over 60 minutes. Thin layer chromatography of whole blood identified a species that moved with the solvent front, corresponding to unbound ^{99m}Tc-pertechnetate, which was excreted in urine. There was no evidence of particle-bound ^{99m}Tc at the origin. Gamma camera images demonstrated high levels of Technegas retention (95.6±1.7% at 6 hours) in the lungs, with no accumulation of radioactivity detected over the liver or spleen. The majority of ^{99m}Tc-labelled carbon nanoparticles remain within the lung up to six hours after inhalation. In contrast to previous published studies, thin layer chromatography did not support the hypothesis that inhaled Technegas carbon nanoparticles pass directly from the lungs into the systemic circulation.

5.2 INTRODUCTION

Epidemiological studies have demonstrated that exposure to air pollution is an important risk factor in the development of cardiovascular disease [Dockery *et al*, 1993; Pope *et al*, 2004; Samet *et al*, 2000]. The WHO identifies particulate matter with an aerodynamic diameter of less than 10 μm (PM_{10}) as the most hazardous component of air pollution. However, only fine particles with a diameter less than 2.5 μm ($\text{PM}_{2.5}$) are able to penetrate to the terminal bronchioles and proximal alveoli to any great extent. Nanoparticles have a diameter of less than 0.1 μm , and whilst constituting a small fraction of the total mass of ambient particulate matter, they represent a substantial proportion in terms of particle number. Toxicologists suggest that the nanoparticulate component of ambient particulate matter is the most potent and likely to be responsible for adverse cardiovascular health effects [Donaldson *et al*, 2001].

The precise mechanism by which air pollution influences cardiovascular risk has not been determined. However, a number of interesting hypotheses have been proposed to explain how inhaled particles could interact with the cardiovascular system [Brook *et al*, 2004]. The traditional view is that inhaled particles provoke an inflammatory response in the lungs, with consequent release of prothrombotic and inflammatory cytokines into the circulation [Seaton *et al*, 1995]. However, a recent publication by Nemmar *et al* suggests that inhaled insoluble nanoparticles may be capable of rapid translocation into the circulation [Nemmar *et al*, 2002], with the potential for direct effects on haemostasis or cardiovascular integrity. They used an aerosol of

Technetium-99m (^{99m}Tc) labelled carbon nanoparticles, 'Technegas', which is used routinely in clinical practice for radionuclide lung ventilation imaging. Primary Technegas particles are 7-23 nm in diameter and aggregate to a mean particle diameter of approximately 100 nm [Lemb *et al*, 1993]. Thin layer chromatography of whole blood identified the presence of ^{99m}Tc -labelled carbon nanoparticles in the bloodstream as early as 1 minute after Technegas inhalation.

Whilst a number of relevant studies have been conducted in animals [Nemmar *et al*, 2001; Oberdorster *et al*, 2002], the work by Nemmar and colleagues is the only study to date that suggests translocation of insoluble inhaled nanoparticulate to the circulation in man. Alternative explanations for Nemmar's findings have been proposed, which focus on the method of Technegas generation (Brown *et al*, 2002; Burch, 2002). In particular, the rapid clearance of ^{99m}Tc from the lungs in this study suggests the presence of large quantities of soluble unbound ^{99m}Tc in both the aerosol and bloodstream. The original study did not report the proportion of ^{99m}Tc remaining in the lung, preventing comment on the importance of this clearance mechanism. As part of a study into the vascular effects of combustion-derived nanoparticulate, we reinvestigate whether inhaled Technegas nanoparticles enter the circulation in man.

5.3 METHODS

5.3.1 Subjects

Ten healthy non-smoking volunteers (5 male, 5 female; aged 21 to 24 years) participated in the study, which was undertaken with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and the written informed consent of each subject. Volunteers were life-long non-smokers with normal lung function and no regular exposure to dust or passive smoke in the workplace. None of the volunteers had infective illnesses or received medication other than oral contraception in the week before the study.

5.3.2 Study design

Immediately after aerosol generation, subjects inhaled approximately 100 MBq of Technegas in 3 breaths via a mouthpiece according to standard clinical procedure [Cook and Clarke, 1992]. Subjects rinsed their mouth with water following inhalation to minimize delivery to the gastrointestinal tract. Blood samples for thin-layer chromatography and measurement of activity in the bloodstream and were collected via a 17 gauge intra-venous cannula into 2.9 mL citrated blood tubes (Sarstedt-Monovette) at 1, 10, 30, 60 and 360 minutes after Technegas inhalation. A urine sample was collected 60 minutes after inhalation. The activity of each sample was measured with a gamma counter (Cobra II Auto-Gamma, Packard Bioscience Company), decay corrected to the time of Technegas inhalation, and expressed as counts per minute per gram of whole blood or urine.

5.3.3 Technegas generation

Technegas was generated using a commercially available generator (Technegas generator, Vita Medical Ltd, Sydney) and the manufacturer's recommended protocol. Briefly, 0.14 mL of sodium $^{99\text{m}}\text{Tc}$ -pertechnetate ($\text{Na}^{99\text{m}}\text{TcO}_4$) solution (2 GBq/mL) was added to a graphite crucible. The solution was evaporated to dryness during the 10 minute simmer phase. The Technegas was then generated during the burn phase in which the crucible was resistively heated at 2550°C for 15 seconds in an atmosphere of 99.998% argon.

To obtain samples for *in vitro* studies, Technegas was drawn through 10 mL of liquid in a conical flask; distilled water for electron microscopy and 0.9% sodium chloride in water for thin-layer chromatography.

5.3.4 Particle sizing

Samples for particle sizing by transmission electron microscopy were prepared by placing one drop of the Technegas suspension on a copper grid coated with a carbon film. The grid was left to dry in air at room temperature. Particles were sized using a transmission electron microscope (JEM-1200EX, JEOL, USA) working at 80 kV.

Particle size distribution was evaluated using an automatic image analyser and Origin software (Microcal, Northampton, USA). The size distribution of aggregates in the aerosol was measured at the outlet of the Technegas generator by a Scanning Mobility Particle Sizer (SMPS, TSI Inc, USA) that consists of a Condensation Particle Counter (CPC) and a Differential Mobility Analyzer (DMA), and can detect aerosol particles in the size range from 7 to 487 nm.

5.3.5 Thin layer chromatography

Thin layer chromatography (TLC) was performed on 20 x 2.5 cm silica gel impregnated glass fibre plates (ITLC-SG, Pall Corporation, USA) with a mobile phase of 0.9% sodium chloride in water. A 50 μ L blood sample was applied 2.5 cm from the bottom of the plate. The plate was placed immediately in the mobile phase and the solvent front was allowed to rise 12 cm. The plate was cut into 1 cm high transverse strips starting 0.5 cm below the origin. This resulted in 13 strips. These were placed into tubes in pairs with only a single strip in the final tube. The count-rate from each tube was measured using an automatic gamma counter (CobraTM II Auto-Gamma, Packard Bioscience Company). Each strip was counted for 10 minutes. The chromatogram was constructed by expressing the count-rate from each tube as a percentage of the total of the count-rates from all seven tubes. A blood sample from one subject was analysed in triplicate to assess repeatability of the thin-layer chromatographic technique.

Control samples consisting of Technegas in 0.9% sodium chloride solution, sodium ^{99m}Tc-pertechnetate solution, whole blood + Technegas solution and whole blood + sodium ^{99m}Tc-pertechnetate solution were prepared and analysed by thin-layer chromatography. The potential for dissociation of the particle-bound ^{99m}Tc in Technegas to unbound ^{99m}Tc-pertechnetate *in vitro* was assessed by thin-layer chromatography of the Technegas sample in 0.9% sodium chloride solution at 1, 60 and 360 minutes after aerosol generation.

5.3.6 Radionuclide imaging

Between 0 and 5 minutes after inhalation of the Technegas, a static image of the thorax was acquired with a GE Millennium gamma camera (GE Millennium gamma camera, GE USA). This was followed by the acquisition of 10 dynamic abdominal images between 5 and 55 minutes. Further 5-minute static thoracic and abdominal images were acquired at 60 and 360 minutes to assess pulmonary retention of ^{99m}Tc .

5.3.7 Data analysis

All results include a correction for background radioactivity and the decay of ^{99m}Tc (half-life = 6.02 hours), with values presented as mean \pm SEM. The total amount of radioactivity in the lung was recorded as total counts from each 5 minute static image taken at 0, 60 and 360 minutes. The bladder, left and right upper quadrants of the abdomen were selected as regions of interest on the dynamic scans. Radioactivity within each region of interest was recorded as counts per pixel from each 5-minute abdominal image and expressed as a percentage of initial lung radioactivity.

5.4 RESULTS

5.4.1 Particle size

A size distribution study of Technegas particles ($n=1,500$) using transmission electron microscopy indicated that the majority of particles were 4-20 nm in diameter (Figure 5.1). There appeared to be a bimodal distribution, where more than two thirds of all particles were less than 10 nm in diameter with a mode of 4 nm, one third were larger with a mode of 15 nm, and rare aggregates were seen of up to 200 nm. Inhaled particles also had a bimodal distribution with the first peak, due to primary particles, increasing exponentially to the lower detection limit of the SMPS at 7 nm. The size of aggregated particles showed a lognormal distribution and typically ranged from 50 to 150 nm, with a count median diameter of 108 nm (geometric standard deviation = 2.2 nm).

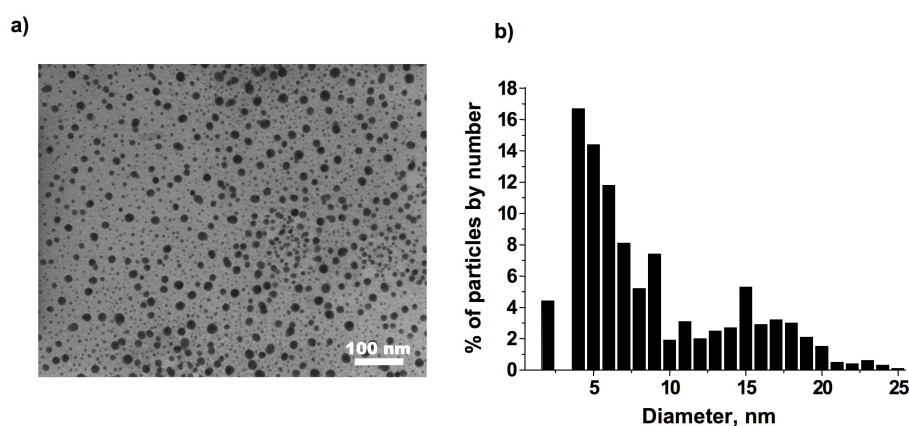


Figure 5.1 (a) Transmission electron micrograph of Technegas particles and (b) a size distribution study of 1,500 Technegas particles.

5.4.2 Thin layer chromatography

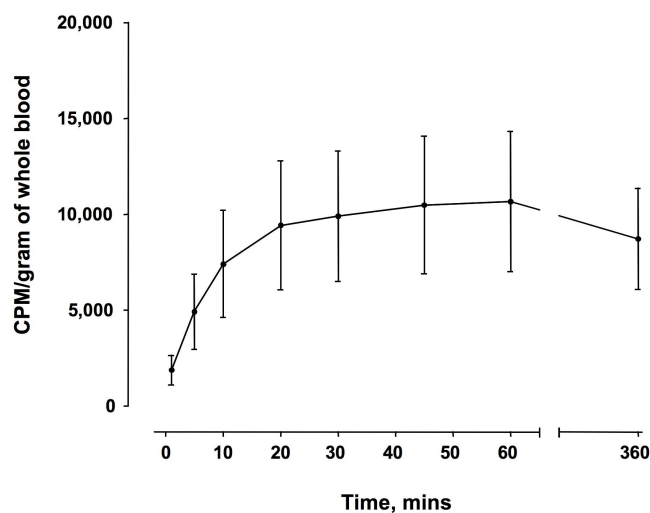


Figure 5.2 Radioactivity measured as counts per minute (CPM) per gram of whole blood at intervals after inhalation of Technegas. Values are mean \pm SEM.

Radioactivity was detected in whole blood within the first minute, reaching a maximum at 60 minutes, and declining thereafter (Figure 5.2). In the chromatograms obtained from blood samples taken at 1, 10, 30, and 60 minutes after Technegas inhalation there is little evidence of ^{99m}Tc at the origin, but a consistent peak trailing the solvent front is seen at all time points (Figure 5.3). The large error bar at the origin, seen at 1 minute, was due to a single outlier. A small peak was present at the origin at 360 minutes.

Chromatography of sodium ^{99m}Tc -pertechnetate solution results in a single peak at the solvent front (Figure 5.4A). Chromatography of Technegas aerosol solution (Figure 5.4B) results in two peaks, one at the origin and one at the solvent front. This is consistent with the presence of both particle-bound ^{99m}Tc and unbound ^{99m}Tc -pertechnetate. The ratio of particle-bound ^{99m}Tc to unbound ^{99m}Tc -pertechnetate did not change over 360 minutes, suggesting that the Technegas aerosol contained both particle bound and unbound ^{99m}Tc at the onset, and that dissociation of ^{99m}Tc from the particles does not occur *in vitro*. A single peak is seen at the solvent front in urine sampled 60 minutes after Technegas inhalation (Figure 5.4C), suggesting that unbound ^{99m}Tc -pertechnetate or a similar species is excreted in the urine.

Peaks are identified at the origin and trailing the solvent front when Technegas aerosol solution was added to whole blood (Figure 5.4D). The addition of sodium ^{99m}Tc -pertechnetate solution to whole blood produces a single peak trailing the solvent front, with no peak at the origin or front (Figure 5.4E). It is probable that this peak is due to ^{99m}Tc -pertechnetate held up by an interaction with one of more components in blood. Replication of chromatography on a single blood sample confirmed good repeatability of our technique (Figure 5.4F).

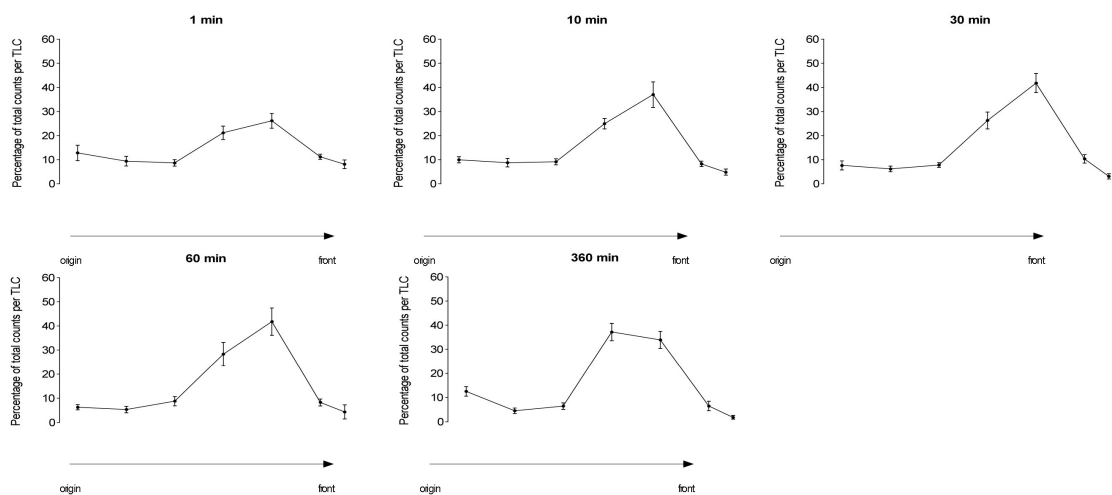


Figure 5.3 Thin-layer chromatograms from whole blood samples taken 1, 10, 30, 60 and 360 minutes after inhalation of Technegas. Values are mean \pm SEM.

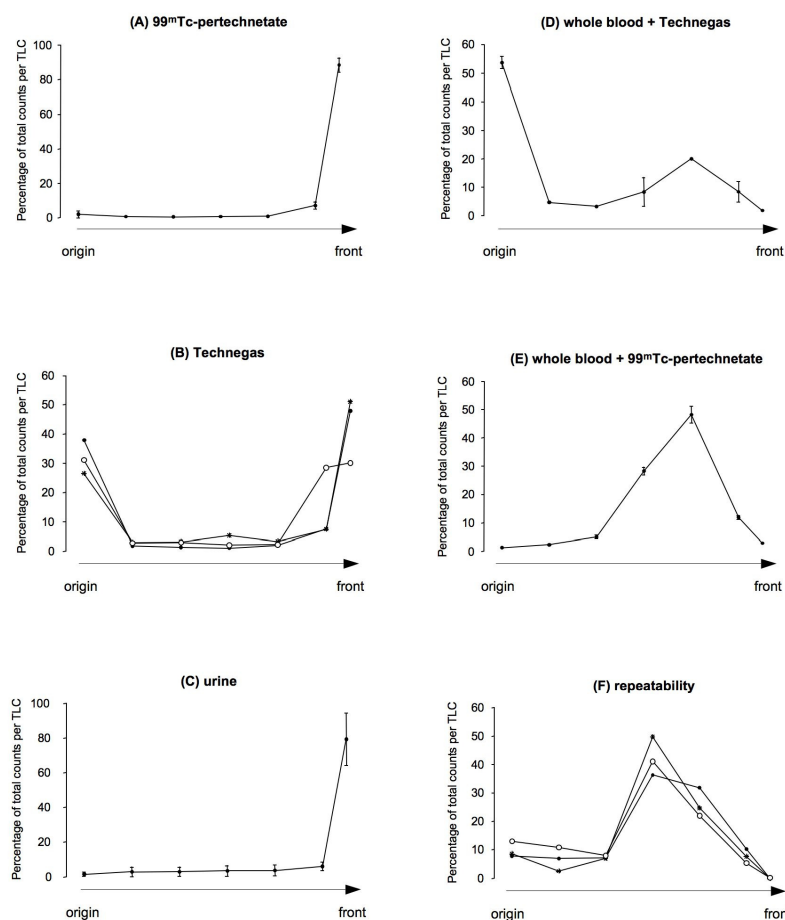


Figure 5.4 Thin-layer chromatography controls: (A) Sodium ^{99m}Tc -pertechnetate, showing a single peak at the solvent front; (B) Technegas aerosol showing two peaks, one at the origin and the other at the solvent front, immediately (\bullet), 60 (\ast) and 360 (\circ) minutes after aerosol collection; (C) Urine sample 60 minutes after inhalation showing a single peak at the solvent front; (D) Technegas aerosol added to whole blood showing peaks at the origin and trailing the solvent front; (E) Sodium ^{99m}Tc -pertechnetate, added to whole blood showing a single peak trailing the solvent front; (F) Replication of chromatography on a single blood sample. Values are mean \pm SEM.

5.4.3 Radionuclide imaging

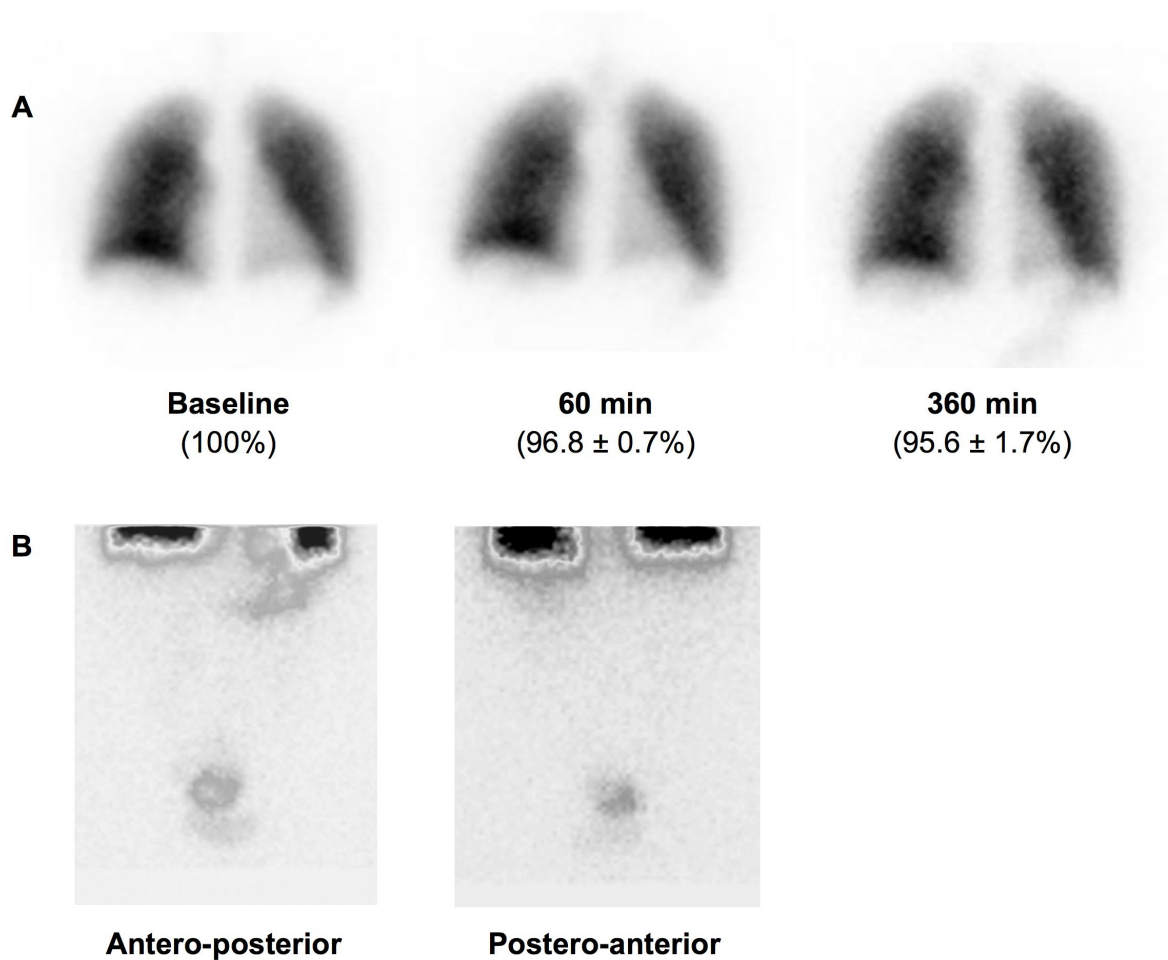


Figure 5.5. (A) Typical antero-posterior lung images at 1, 60 and 360 minutes after inhalation of Technegas. Values of retention are mean \pm SEM. (B) Typical antero-posterior and postero-anterior abdominal images recorded at 360 minutes post inhalation of Technegas.

The average decrease in lung radioactivity was $3.2 \pm 0.7\%$ over the first hour, and $1.2 \pm 1.7\%$ over the subsequent 5 hours (Figure 5.5A). Radioactivity was present within the gastrointestinal tract and bladder, but no obvious uptake of ^{99m}Tc by the liver or spleen was detected (Figure 5.5B). The level of radioactivity over the bladder region gradually increased with time, but did not accumulate in any other region (Figure 5.6).

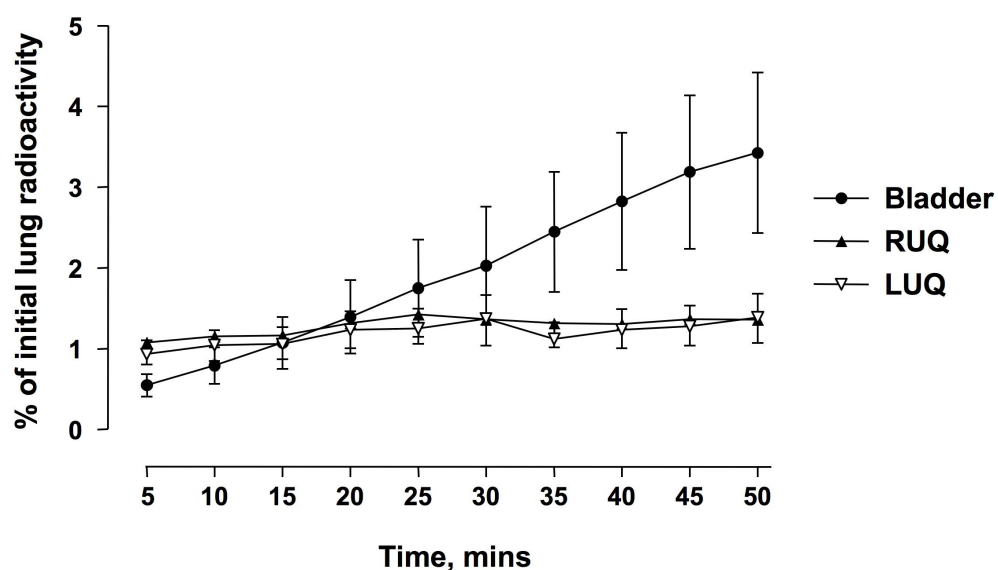


Figure 5.6. Time-activity curve over bladder, right and left upper quadrants expressed as percent initial lung radioactivity. Values are mean \pm SEM.

5.5 DISCUSSION

Air pollution has been consistently linked to adverse cardiovascular events [Dockery *et al*, 1993; Pope *et al*, 2004; Samet *et al*, 2000], however the mechanism responsible for this association is not well understood. Rapid translocation of inhaled nanoparticles into the systemic circulation with direct effects on the vasculature could potentially explain this association with cardiovascular risk. However, using ^{99m}Tc -radiolabelled carbon nanoparticles, we found no evidence to support this hypothesis.

Following inhalation of Technegas, ^{99m}Tc was immediately detected in blood, with levels increasing steadily over the first 60 minutes. Thin layer chromatography of whole blood identified a species that moved with the solvent, corresponding to unbound ^{99m}Tc -pertechnetate, but no evidence of significant particle-bound ^{99m}Tc at the origin. Gamma camera images demonstrated high levels of ^{99m}Tc retention ($96.8 \pm 0.7\%$ at 1 hour) in the lungs. Losses were explained by the accumulation of ^{99m}Tc in the bladder ($3.4 \pm 1.0\%$), which was confirmed as ^{99m}Tc -pertechnetate by chromatography of urine.

Nemmar *et al* also detected ^{99m}Tc in the bloodstream immediately following inhalation of Technegas. In contrast, the majority of ^{99m}Tc remained at the origin of the chromatogram, suggesting the presence of circulating particle-bound ^{99m}Tc . In addition, substantial quantities of radioactivity were measured over the liver and other extra-thoracic organs. The authors concluded that inhaled particle-bound ^{99m}Tc

rapidly translocates into the circulation with particulate accumulating in the endoreticular system. Differences in aerosol composition, radionuclide imaging and chromatography between our study and that of Nemmar *et al* merit further discussion.

In the presence of even minute quantities of oxygen, the Technegas generator produces a mixed aerosol of ^{99m}Tc labelled particles and soluble oxides of ^{99m}Tc -pertechnetate (Pertechnegas). Therefore the presence of ^{99m}Tc activity in the bloodstream or over extra-thoracic organs does not in itself provide evidence of particle translocation. Pertechnegas is cleared from the lungs with a half-life of approximately 10 minutes [Mackey *et al*, 1997], and has been used clinically to assess lung epithelial permeability [Monaghan *et al*, 1991]. Pertechnegas and the free radiolabel ^{99m}Tc -pertechnetate behave similarly on chromatography [Tominaga *et al*, 1995], have identical clearance kinetics following inhalation [Isawa *et al*, 1995], and identical distributions following intravenous infusion [Tominaga *et al*, 1995]. The six hour retention of approximately 95% of inhaled ^{99m}Tc in our study is consistent with rates quoted by the manufacturer [Burch, 2002] and previous independent studies [Brown *et al*, 2002]. The loss of 5% of ^{99m}Tc over and above decay occurred within the first hour, with little further loss over the remaining five hours. The time course of this clearance pattern is highly suggestive of the diffusion of small quantities of Pertechnegas into the bloodstream.

Abdominal images demonstrate a clear increase in radioactivity in the bladder, which accounted for the loss of lung radioactivity after 60 minutes. Chromatography

confirmed that the activity in the bladder was due to ^{99m}Tc -pertechnetate in the urine. The presence of soluble ^{99m}Tc in the Technegas aerosol, the blood stream and in urine, strongly suggest that a small amount of ^{99m}Tc in the aerosol produced by our generator was in the form of ^{99m}Tc -pertechnetate or Pertechnegas, which diffused rapidly into the circulation and was eliminated via the renal tract.

In the study by Nemmar *et al*, radioactivity over the bladder was present early and increased steadily with as much as 25% of total initial lung radioactivity present in the bladder at 45 minutes; chromatography confirmed that this activity was due to ^{99m}Tc -pertechnetate in urine. In addition, 10% of the initial lung radioactivity was present in the liver after 5 minutes and there were large quantities of radioactivity visible and unaccounted for in the thyroid, salivary glands and stomach. From their data, we can infer that pulmonary retention of ^{99m}Tc after 45 minutes would be no more than 65% although this value was not formally reported. This is in stark contrast to previous studies and the present study, and led to the highly plausible suggestion from one correspondent that large quantities of soluble ^{99m}Tc had been produced by a generator contaminated with oxygen [Burch, 2002].

Given the very low pulmonary retention rates and the rapid accumulation of ^{99m}Tc -pertechnetate in urine, it is surprising that chromatography of the aerosol in Nemmar's study suggests it consisted only of ^{99m}Tc -labelled particles with no ^{99m}Tc -pertechnetate or Pertechnegas. Despite using the same generator and purity of argon, this was not the case in our study or in a previous study [Scalzetti and Gagne, 1995]. Scalzetti and Gagne used thin layer chromatography to investigate the effect of

increasing the ratio of oxygen: argon in the generator on the transition of Technegas to Pertechnegas. When using the recommended highly purified argon (99.998% purity) as in our study, they still found approximately 20% of the ^{99m}Tc migrated with the solvent front. In oxygen concentrations of 0.1% or more an abrupt transition from Technegas to Pertechnegas occurred with more than 50% of the ^{99m}Tc moving with the solvent front [Scalzetti and Gagne, 1995].

Approximately 5% of inhaled ^{99m}Tc in our study was in the form of unbound ^{99m}Tc -pertechnetate and diffused rapidly into the circulation before elimination via the renal tract. Thin-layer chromatography identified this small quantity of ^{99m}Tc -pertechnetate in the blood stream as a consistent and reproducible peak trailing the solvent front. In the study by Nemmar *et al*, 25% of ^{99m}Tc was filtered by the kidneys within the first 45 minutes, yet chromatography did not reliably identify unbound ^{99m}Tc -pertechnetate in the blood stream; the largest peak occurred at the origin and was ascribed to particle bound ^{99m}Tc . To explain the lack of consistency between chromatograms of the aerosol, blood and urine, Nemmar *et al* suggest that soluble oxides of ^{99m}Tc -pertechnetate may be being produced *in vivo* [Nemmar *et al*, 2002]. Whilst plausible there was no evidence of *in vitro* oxidation in either Nemmar's or our own control studies.

It is possible that differences in chromatography technique could explain this disparity. Nemmar *et al* dried the blood sample at the origin of the plate before running the chromatogram (Dr A Nemmar, personal communication), whilst we placed it immediately into the mobile phase. It is possible that a period of drying

would favour ^{99m}Tc retention at the origin. Interestingly, in our study there was a small but reproducible peak of activity at the origin in blood sampled 6 hours after inhalation. This may reflect particle bound activity due to translocation via a different mechanism involving a slower active transfer of particles from the lung into the circulation. Alternatively, the activity may be intracellular due to phagocytosis of particulate in the lung alveolar space or interstitium by cells of the monocyte/macrophage lineage, which then enter the circulation. Further studies are required to determine the significance of this finding. Thin layer chromatography is an indirect method of particle detection, and ideally, a more direct strategy could be employed to track particles in the bloodstream. Techniques such as electron microscopy would be limited by the small concentration of particles, difficulty resolving structures in the nanoparticulate range, and the low electron density of carbon.

In the study by Nemmar *et al*, 10% of the initial lung radioactivity was present in the liver after 5 minutes, which the authors suggest is due to the accumulation of circulating particles in the Kupffer cells. If most of the circulating ^{99m}Tc were particle bound and cleared in this way then radioactivity should increase over time in the liver. However, it did not, but rather increased steadily in the bladder. It is possible that the large amount of activity measured over the liver was not associated with the liver and simply reflected scatter from the lung. An alternative explanation could relate to the vascularity of this organ. If there is a large quantity of circulating unbound ^{99m}Tc , either as Pertechnegas from a generator contaminated with oxygen or from oxidation *in vivo*, then radioactivity over highly vascular organs might well

be high. Furthermore, it would remain static, as hepatic blood flow is unlikely to change during the course of the study, or even decrease, as $^{99\text{m}}\text{Tc}$ -pertechnetate is filtered by the kidneys.

In our study, the only discernable pattern of radioactivity across the abdomen, outwith the bladder, was suggestive of gastrointestinal uptake, which is likely to be due to either mucociliary clearance of particles from the bronchial tree or swallowing of particles that had deposited in the mouth. The small quantities of radioactivity detected in the left and right upper quadrants likely reflect scatter from the lung or overlap of the lung and liver parenchyma. This absence of appreciable radioactivity in the liver or spleen is consistent with previous animal [Kreyling *et al*, 2002] and human studies [Brown *et al*, 2002]. There were small amounts of activity in the thyroid and stomach at 60 and 360 minutes after inhalation, both glandular organs in which $^{99\text{m}}\text{Tc}$ -pertechnetate is known to localise.

The ability of nanoparticles to cross the lung-blood barrier is likely to be influenced by particle size. Differences in size of the primary particle and in the degree of particle aggregation could potentially explain the differences between study results. In our aerosol the majority of particles were singlet and less than 10 nm in diameter, but these rapidly formed aggregates of approximately 100 nm in diameter in the inhaled aerosol. Nemmar and colleagues identified 5 nm particles, but the average particle size and the size distribution of aggregated particles in the inhaled aerosol was not reported. During our study, a standard protocol for generating and

administering Technegas was followed, making it unlikely that particles would aggregate to a greater or lesser extent than in previous studies.

The escape of nanoparticulate from the lung into the bloodstream has been advanced as a mechanism for the adverse cardiovascular effects of air pollution, whilst ‘engineered’ nanoparticles in the nanotechnology industry could pose the same risks [Donaldson *et al*, 2004]. The fact that Technegas nanoparticles do not translocate directly into the blood in this study does not necessarily mean that other nanoparticles behave similarly, nor does it rule out an interaction between inhaled particulate and the vasculature. Environmental combustion derived nanoparticulate, as a carrier of soluble organic compounds from unburned hydrocarbon fuels [Levsen, 2002], and oxidized transition metals [Sydbom *et al*, 2001], may well exert an important influence on the cardiovascular system.

In conclusion, our results suggest that small quantities of soluble ^{99m}Tc species, not ^{99m}Tc -radiolabelled nanoparticles, are responsible for the increase in radioactivity found in the bloodstream following Technegas inhalation. Robust chromatography and clear radio-nuclear imaging provide support for this statement. The low pulmonary retention rates of ^{99m}Tc and whole body images reported in Nemmar *et al* are suggestive of a high degree of Pertechnegas contamination. However, thin layer chromatographic analysis of their aerosol, blood and urine are not entirely consistent with this, and in light of our findings are difficult to explain. A better knowledge of the toxicokinetics of nanoparticulate is required before we can make general

conclusions about the behaviour of other inhaled environmental and engineered nanoparticles.

CHAPTER 6

ISCHEMIC AND THROMBOTIC EFFECTS OF DILUTE DIESEL EXHAUST INHALATION IN MEN WITH CORONARY HEART DISEASE

Published by Mills NL, Törnqvist H, Gonzales M, Fink E, Robinson SD, Boon NA, Donaldson K, Blomberg A, Sandström T, and Newby DE. Ischemic and thrombotic effects of dilute diesel exhaust inhalation in men with coronary heart disease. *N Engl J Med* 2007;**357**:1075-1082.

6.1 SUMMARY

Exposure to traffic-derived air pollution is associated with adverse cardiovascular events. The mechanisms for this association are unknown. We conducted a controlled exposure to dilute diesel exhaust in patients with stable coronary heart disease to determine the direct effect of air pollution on myocardial, vascular and fibrinolytic function. In a double blind randomized cross-over study, 20 men with prior myocardial infarction were exposed to dilute diesel exhaust ($300\mu\text{g}/\text{m}^3$) or filtered air for one hour during periods of rest and moderate exercise in a controlled exposure facility. During the exposure, myocardial ischemia was quantified by ST-segment analysis using continuous 12-lead electrocardiography. Six hours following exposure, vascular vasomotor and fibrinolytic function were assessed by means of intra-arterial agonist infusions. During both exposures, heart rate increased with exercise ($P<0.001$ for both) to a similar extent ($P=0.67$; diesel exhaust *versus* filtered air). Exercise induced ST-segment depression was present in all patients but there was a greater increase in ischemic burden during exposure to diesel exhaust (-22 ± 4 *versus* -8 ± 6 mVs, $P<0.001$). Exposure to diesel exhaust did not aggravate pre-existing vasomotor dysfunction, but did reduce acute endothelial tissue plasminogen activator release ($P<0.05$; area under the curve decreased by 35%). Brief exposure to dilute diesel exhaust promotes myocardial ischemia and inhibits endogenous fibrinolytic capacity in men with stable coronary heart disease. Our findings have identified ischemic and thrombotic mechanisms that may explain in part the observations that exposure to combustion-derived air pollution is associated with adverse cardiovascular events.

6.2 INTRODUCTION

The WHO estimates that air pollution is responsible for 800,000 premature deaths worldwide each year [World Health, 2004]. Short-term exposure to air pollution has been associated with increases in cardiovascular morbidity and mortality with deaths due to ischemia, arrhythmia and heart failure [Brook *et al*, 2004]. Miller *et al* recently reported that long-term exposure to air pollution increases the risk of death from cardiovascular disease by 76% [Miller *et al*, 2007]. These associations are strongest for PM_{2.5}, of which combustion-derived nanoparticulate in diesel exhaust is an important component [Laden *et al*, 2000]. Significant improvements in air quality have occurred over the last 50 years, yet the association between PM_{2.5} and mortality has no apparent threshold and is evident below current air quality standards [Ware, 2000].

Pre-clinical models of exposure to particulate air pollution demonstrate accelerated atherosclerotic plaque development [Sun *et al*, 2005a] and increased *in vitro* [Radomski *et al*, 2005] and *in vivo* [Nemmar *et al*, 2004] platelet aggregation. Epidemiologic and observational clinical studies suggest that exposure to air pollution may worsen symptoms of angina, [von Klot *et al*, 2005] exacerbate exercise induced myocardial ischemia [Gold *et al*, 2005; Pekkanen *et al*, 2002], and trigger acute myocardial infarction [Peters *et al*, 2001a; Peters *et al*, 2004]. These clinical findings are limited by imprecision in the measurement of pollution exposure, the effect of potential confounding environmental and social factors, and the lack of mechanistic data [Stone, 2004]. Controlled exposures of air pollutants can

help to address these shortcomings by providing a precisely defined exposure in a regulated environment that facilitates investigation with validated biomarkers and surrogate measures of cardiovascular health. Using a carefully characterised exposure system, we have previously shown in healthy volunteers that exposure to dilute diesel exhaust causes lung inflammation [Salvi *et al*, 1999], depletion of airway antioxidant defences, [Behndig *et al*, 2006] and impairment of vascular and fibrinolytic function [Mills *et al*, 2005]. To date, there have been no controlled exposures in patients with coronary heart disease: an important population who may be particularly susceptible to the adverse cardiovascular effects of air pollution.

We have therefore assessed the effect of dilute diesel exhaust inhalation on myocardial, vascular and fibrinolytic function in an “at risk” population of patients with stable coronary heart disease.

6.3 METHODS

6.3.1 Subjects

Twenty patients with stable coronary artery disease participated in this study that was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of all volunteers.

All patients had proven coronary heart disease with a previous myocardial infarction (>6 months previously) treated by primary angioplasty and stenting, and were receiving standard secondary preventative therapy. Patients with angina pectoris (Canadian Cardiovascular Society grade ≥ 2), a history of arrhythmia, diabetes mellitus, uncontrolled hypertension, renal or hepatic failure, or those with unstable coronary disease (acute coronary syndrome or unstable symptoms within 3 months) were excluded. All volunteers were invited to a pre-study screening visit for exercise stress testing and patients unable to achieve stage 2 of the Bruce protocol, or patients who had marked ECG changes (left bundle branch block, early ST depression >2mm) or developed hypotension were excluded. Current smokers and those with asthma, significant occupational exposure to air pollution, or an inter-current illness were also excluded from the study.

6.3.2 Study design

Using a randomized double blind cross-over study design, volunteers attended at 08.00 hours on two occasions at least two weeks apart for controlled exposure to

dilute diesel exhaust or filtered air. Each subject was exposed for one hour in an exposure chamber as previously described [Salvi *et al*, 1999]. During each exposure, subjects performed two 15-min periods of exercise on a bicycle ergometer separated by two 15-min periods of rest. For each subject, the ergometer workload was titrated to achieve a minute ventilation of 15 L/min/m² to ensure a similar exposure. The workload was constant for both exposures and was equivalent to Stage 2 of the Bruce Protocol (range 110-150 Watts, 5-7 METS). All volunteers were fitted with 12-lead Holter electrocardiographic monitors (Reynolds Medical Lifecard 12, Delmar Reynolds, UK). Based on previous exposure studies in healthy volunteers, vascular assessments were performed 6-8 hours following diesel or air exposure [Mills *et al*, 2005].

6.3.3 Diesel Exposure

The diesel exhaust was generated from an idling Volvo diesel engine (Volvo TD45, 4.5L, 4 cylinders, 680rpm) from Swedish Low Sulphur Gasoil E10 (Preem, Göteborg, Sweden) as described previously [Salvi *et al*, 1999]. Over 90% of the exhaust was shunted away, and the remainder diluted with filtered air heated to 20°C (humidity ~50%) before being fed into a whole body exposure chamber (3x3x2.4m) at a steady-state concentration.

The chamber was continuously monitored for pollutants with exposures standardised using NO_x concentrations to deliver a PM concentration of 300µg/m³ (median diameter 54nm; range 20-120). There was little variation in particle number (1.26±0.01x10⁶ particles/cm³), NO_x (4.45±0.02ppm), NO₂ (1.01±0.01ppm), NO

(3.45 ± 0.03 ppm), CO (2.9 ± 0.1 ppm) and total hydrocarbon (2.8 ± 0.1 ppm) concentrations between exposures. The predominant PAH (~90%) were phenanthrene, fluorene, 2-methylfluorene, dibenzothiophene and different methyl-substituted phenanthrenes. Only a minor fraction of PAH (3.5%) was associated with PM: 0.04% total PM and 0.06% PM organic fraction. PM₁₀ and NO₂ concentrations in the exposure chamber were 6- and 10-fold greater than WHO air quality standards of $50 \mu\text{g}/\text{m}^3$ and 0.105 ppm respectively [World Health Organisation, 2000].

6.3.4 Vascular studies

All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle. Following a 30min baseline saline infusion, acetylcholine at 5, 10 and $20 \mu\text{g}/\text{min}$ (endothelium-dependent vasodilator; Clinalfa AG, Switzerland), bradykinin at 100, 300 and $1000 \text{ pmol}/\text{min}$ (endothelium-dependent vasodilator that releases tissue plasminogen activator; Clinalfa AG) and sodium nitroprusside at 2, 4 and $8 \mu\text{g}/\text{min}$ (endothelium-independent vasodilator; David Bull Laboratories, UK) were infused for 6min at each dose. The three vasodilators were separated by 20min saline infusions and given in a randomised order. ACE inhibitor therapy was withdrawn 7 days prior to each vascular study as it augments bradykinin induced endothelial t-PA release [Witherow *et al*, 2002]. All other medications were continued throughout the study.

Forearm blood flow was measured in both arms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [Newby *et al*, 1999]. Supine heart rate and blood pressure in the non-infused arm

were monitored at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer.

6.3.5 Fibrinolytic and Inflammatory Markers

Blood (10mL) was withdrawn into acidified buffered citrate (Stabilyte tubes, Biopool International) for t-PA assays, and citrate (BD Vacutainer) for PAI-1 assays. Plasma t-PA and PAI-1 antigen concentrations were determined by enzyme linked immunosorbant assays (TintElize t-PA, Biopool EIA; Coaliza PAI-1, Chromogenix AB). Serum CRP concentrations were measured using an immunonephelometric assay (Behring BN II nephelometer, Marburg, Germany).

6.3.6 Data analysis and statistics

Electrocardiographic recordings were analysed using the Reynolds Medical Pathfinder Digital 700 Series Analysis System (Delmar Reynolds, United Kingdom). ST-segment deviation was calculated by comparing the ST-segment during each 15-min exercise test with the average ST-segment for the 15-min immediately prior to the start of the exposure. The ST-segment amplitude was determined at the J-point plus 80ms. The ischemic burden during each exercise test was determined as the product of the change in ST-segment amplitude and the duration of exercise. Leads II, V₂, and V₅ were selected *a priori* for ST-segment analysis to reflect separate regions of myocardium. Maximum ST-depression and ischaemic burden were determined for these leads individually and as a composite.

Plethysmographic data and net t-PA release were determined as described previously [Newby *et al*, 1997]. Continuous variables are reported as mean \pm SEM. Statistical analyses were performed with GraphPad Prism (Graph Pad Software, USA) using ANOVA with repeated measures and two-tailed Student's *t*-test where appropriate. Statistical significance was taken at a two-sided $P < 0.05$. All authors were involved in study design, data collection, and analysis, and in the decision to submit the paper for publication.

6.4 RESULTS

Subjects were all middle-aged males with predominantly single vessel coronary artery disease (Table 6.1). They reported no symptoms of angina and had no major arrhythmias during, or in the 24 hours following, exposure.

Table 6.1 Baseline characteristics of patients with coronary heart disease (n=20)

Age (years)	60 ± 1
Cigarette smokers (Non-/Ex-/Current)	12/8/0
Hypertension	8
Height (cm)	173 ± 6
Weight (kg)	79 ± 3
Body mass index (m ² /kg)	27 ± 1
Time since index infarction, months	35±4
Coronary angiogram	
No of diseased vessels	
1	13
2	6
3	1
Culprit lesion	
LAD	14

Circumflex	4
RCA	2
Lipid profile	
Total cholesterol (mg/dL)	173 ± 6
LDL-cholesterol (mg/dL)	100 ± 8
HDL-cholesterol (mg/dL)	48 ± 2
Triglycerides (mg/dL)	128 ± 23
Fasting glucose (mg/dL)	102 ± 6
Drugs	
Aspirin	20
Statin	18
β-blocker	15
ACE inhibitor*/ARB	4

Values are presented as number or mean ± SEM;

LAD=left anterior descending artery;

RCA=right coronary artery;

LDL=low density lipoprotein;

HDL=high density lipoprotein;

ACE=Angiotensin converting enzyme;

ARB=angiotensin receptor blocker

*ACE inhibitor therapy was withdrawn 7 days prior to each vascular study. All other regular medications were continued throughout the study.

6.4.1 Myocardial Ischemia

Heart rate increased with exercise during diesel exhaust and filtered air exposures respectively ($P<0.001$, for both *versus* baseline; $P=0.67$, diesel exhaust *versus* filtered air exposure; Table 6.2). Myocardial ischemia was detected during exercise in all patients with greater maximum ST-segment depression during exposure to diesel exhaust than filtered air (Table 6.2, Figure 6.1A and B, $P<0.05$). The ischemic burden induced by exercise was greater during exposure to diesel exhaust (Figure 6.1C, $P<0.001$).

Table 6.2 Effect of exercise on heart rate and ST-segment during filtered air and diesel exhaust exposures

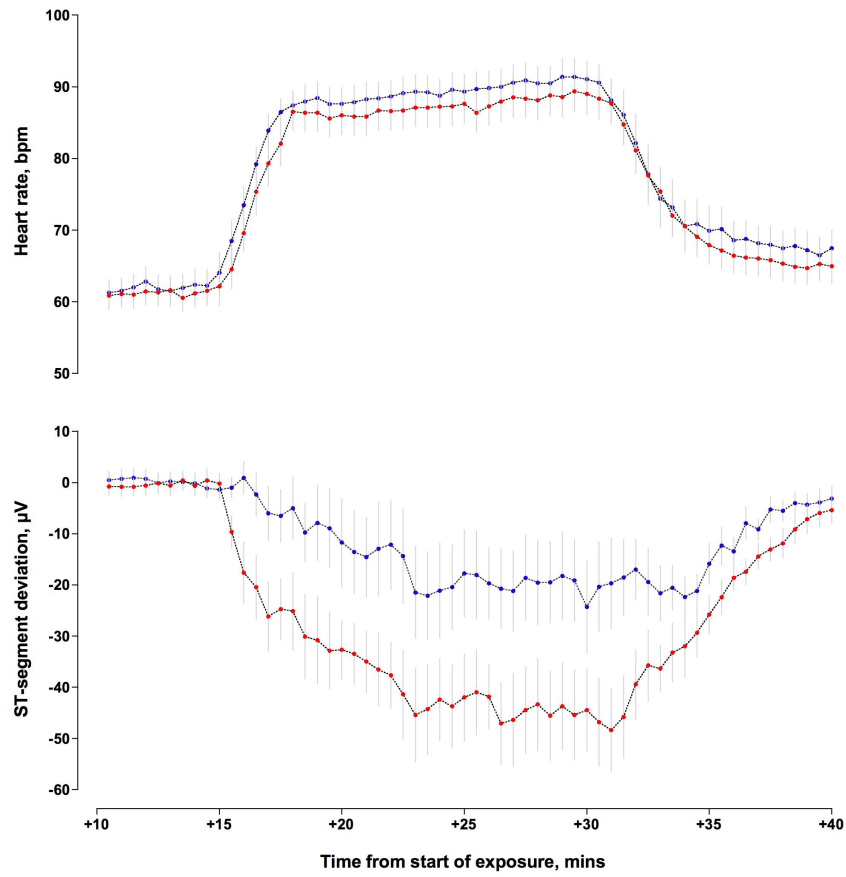
	Air	Diesel	P value
Exercise phase 1			
Baseline HR, bpm	63 ± 2	61 ± 2	0.24
Maximum HR, bpm	87 ± 3	86 ± 3	0.67
Maximum ST Δ , μV			
Lead II	-28 ± 13	-56 ± 10	0.03
Lead V ₂	-28 ± 10	-41 ± 12	0.18
Lead V ₅	-14 ± 8	-33 ± 9	0.04
Ischemic burden, mVs			
Lead II	-11 ± 5	-23 ± 4	0.004
Lead V ₂	-13 ± 5	-21 ± 6	0.04

Lead V ₅	-4 ± 3	-12 ± 4	0.01
Exercise phase 2			
Baseline HR, bpm	67 ± 2	65 ± 2	0.35
Maximum HR, bpm	91 ± 3	87 ± 3	0.12
Maximum ST Δ, μV			
Lead II	-17 ± 15	-49 ± 12	0.006
Lead V ₂	-18 ± 12	-41 ± 13	0.04
Lead V ₅	-7 ± 9	-28 ± 10	0.02
Ischemic burden, mVs			
Lead II	-8 ± 6	-22 ± 4	0.0007
Lead V ₂	-11 ± 5	-20 ± 6	0.02
Lead V ₅	-2 ± 3	-12 ± 5	0.006

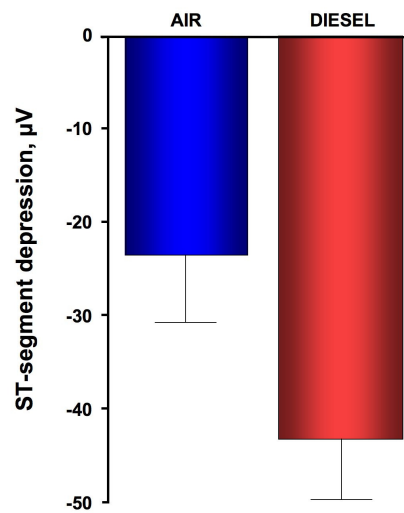
Values are mean ± SEM;

Student's *t*-test diesel exhaust *versus* filtered air (n=20)

a)



b)



c)

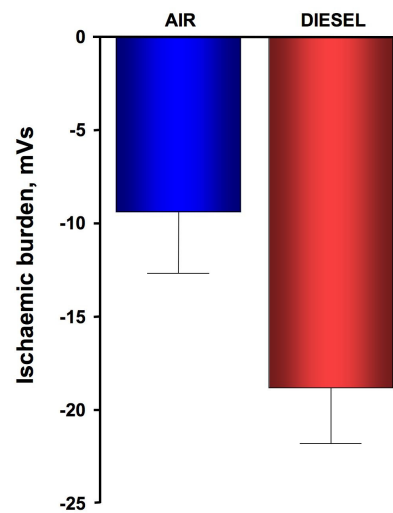


Figure 6.1 Myocardial ischemia during 15-min of exercise stress (period 1: +15 to +30 min) whilst exposed to diesel exhaust (red) or filtered air (blue). (A) Average change in heart rate and ST-segment in lead II. (B) Maximal ST-segment depression ($P=0.003$, diesel exhaust *versus* filtered air), and (C) total ischemic burden ($P<0.001$, diesel exhaust *versus* filtered air) as an average of leads II, V₂, and V₅ (n=20).

6.4.2 Vasomotor Function

There were no differences in resting heart rate, blood pressure or baseline non-infused forearm blood flow between or during the two study visits. Although there was a dose-dependent increase in blood flow with each vasodilator ($P<0.001$ for all), neither endothelium-dependent nor independent vasodilation was affected by inhalation of diesel exhaust (Figure 6.2). Comparison with a contemporary reference population of healthy male volunteers (age 53 ± 4 years), demonstrated impaired vasodilatation to acetylcholine but not sodium nitroprusside-induced vasodilatation ($P<0.05$, patients *versus* controls; Figure 6.2).

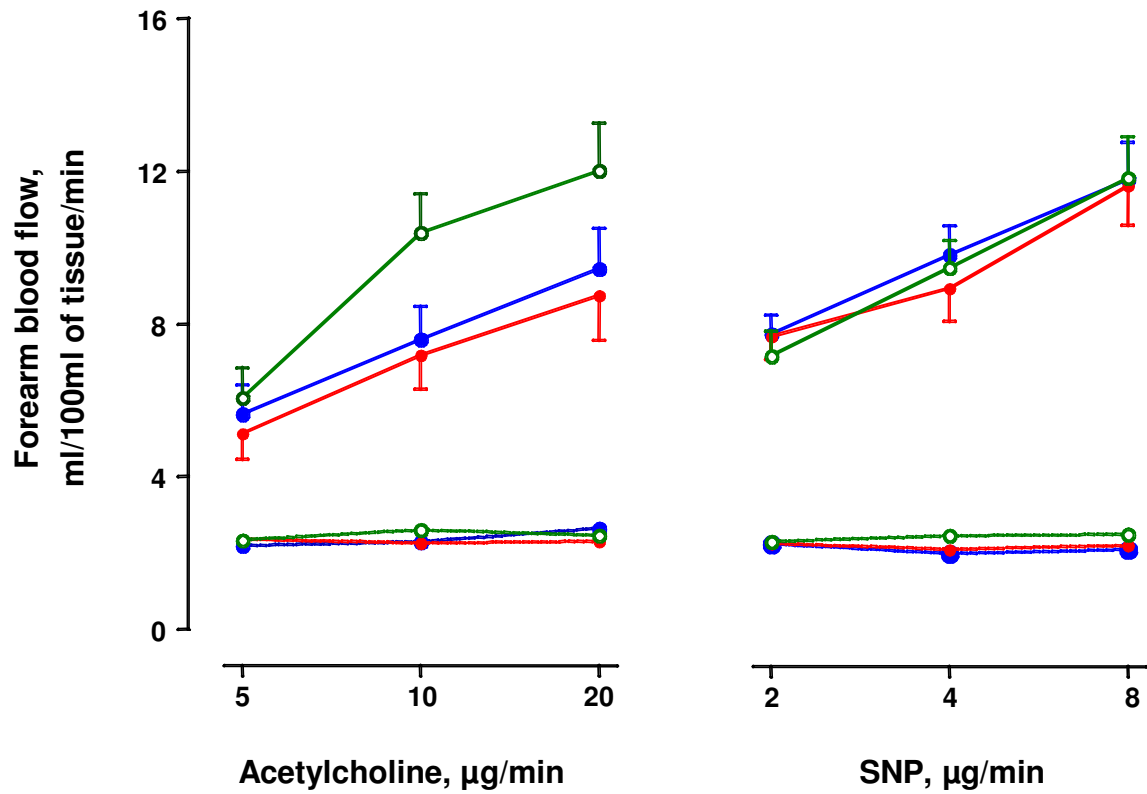


Figure 6.2 Infused (solid line) and non-infused (dashed line) forearm blood flow in patients 6-8 hours following diesel exposure (red) and filtered air (blue), and a reference matched healthy control population (green) during intra-brachial infusion of acetylcholine and sodium nitroprusside (SNP). For all dose responses in the infused arm, $P < 0.0001$. For diesel exposure *versus* air, $P = 0.54$ for acetylcholine and $P = 0.56$ for sodium nitroprusside ($n = 17$). For patients *versus* healthy controls, $P < 0.05$ for acetylcholine and $P = 0.72$ for sodium nitroprusside.

6.4.3 Fibrinolytic and Inflammatory Markers

There were no differences in basal plasma concentrations of t-PA (10.5 ± 1.0 *versus* 9.5 ± 1.0 ng/mL) or its endogenous inhibitor, PAI-1 (18.8 ± 3.0 *versus* 17.0 ± 2.0 ng/mL), six hours following either exposure. Likewise leukocyte, neutrophil and platelet count, and serum CRP concentrations were not altered at 6 or 24 hours by diesel or air exposure. Bradykinin caused a dose-dependent increase in plasma t-PA concentrations (data not shown) and net t-PA release (Figure 6.3) in the infused arm ($P < 0.001$ for both) that was suppressed ($P < 0.05$, area under the curve decreased by 35%) following exposure to diesel exhaust.

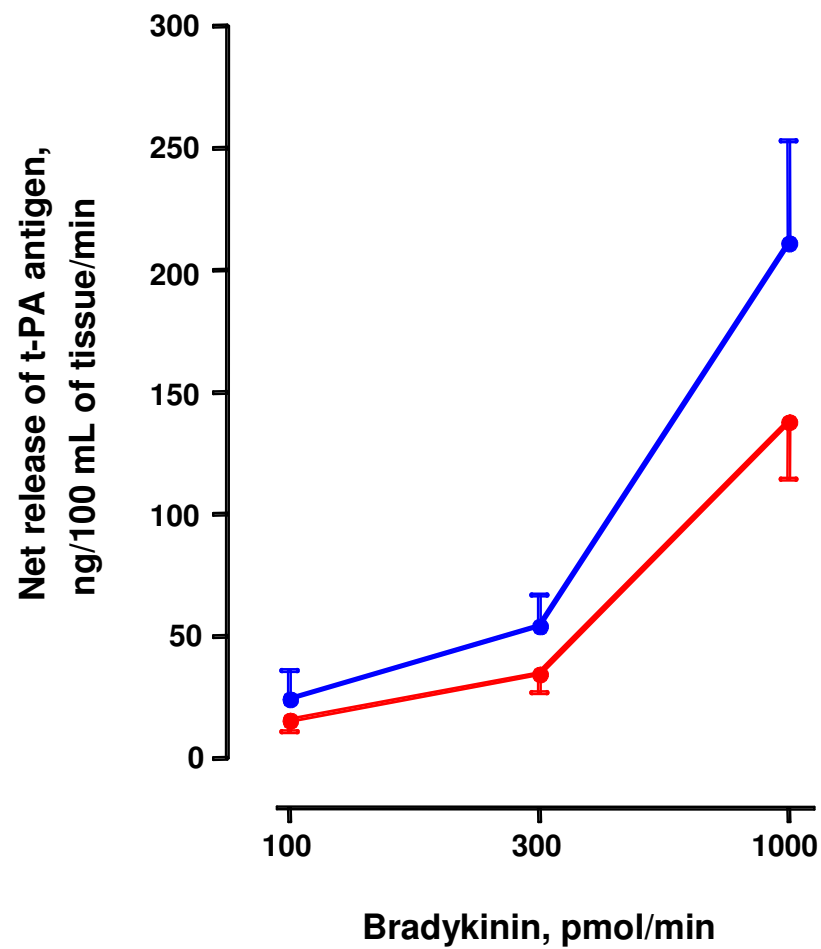


Figure 6.3 In comparison to filtered air (blue), diesel exhaust inhalation (red) reduced net t-PA release (product of forearm plasma flow and the concentration difference between the two arms) by 35% ($P < 0.05$, diesel exhaust *versus* filtered air) 6-8 hours following exposure ($n=17$).

6.5 DISCUSSION

We have demonstrated that transient exposure to dilute diesel exhaust, at concentrations occurring in urban road traffic, exacerbates exercise-induced myocardial ischemia and impairs endogenous fibrinolytic capacity in men with coronary heart disease. These findings provide a plausible explanation for the epidemiologic observations that exposure to air pollution is associated with adverse cardiovascular events.

Particulate matter concentrations can regularly reach levels of $300\mu\text{g}/\text{m}^3$ in heavy traffic, occupational settings, and in the world's largest cities [Report, 1994]. A major proportion of this mass is attributable to combustion-derived nanoparticles from traffic; ranging from 20% at remote monitoring sites [Lanki *et al*, 2006] up to 70% in a road tunnel [Geller *et al*, 2005]. Exposure to $300\mu\text{g}/\text{m}^3$ for one hour increases a person's average exposure over a 24-hour period by only $12\mu\text{g}/\text{m}^3$. Changes of this magnitude occur on a daily basis even in the least polluted cities, and are associated with increases in cardiorespiratory mortality [Samet *et al*, 2000]. Our model is therefore highly relevant both in the composition and magnitude of exposure for the assessment of short-term health effects in man.

Given potential safety concerns, we recruited patients who had stable and symptomatically well-controlled coronary heart disease with good exercise tolerance on formal stress testing. Volunteers were closely monitored throughout the exposure and reported no adverse effects. Despite similar changes in heart rate, we

documented asymptomatic myocardial ischemia that was increased up to three-fold by diesel exhaust inhalation. This reproducible effect was present despite a high use of maintenance β -blocker therapy in patients without limiting angina. Thus we have established an immediate pro-ischemic effect of diesel exhaust inhalation, and we believe this provides an important mechanism for the observed increase in myocardial infarction in the hour following exposure to traffic [Peters *et al*, 2004].

Small areas of denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are usually sub-clinical. Rosenberg and Aird have postulated that vascular bed-specific defects in hemostasis exist, and that propagation of coronary thrombosis is critically dependent upon the local fibrinolytic balance [Rosenberg and Aird, 1999]. The efficacy of endogenous fibrinolysis is determined by the local release of t-PA from the vascular endothelium. The magnitude and rapidity of t-PA release regulates the generation of plasmin and therefore determines the effective removal of intravascular thrombus.

We have previously reported impaired acute t-PA release 6 hours following diesel exhaust inhalation in healthy volunteers although this effect was not seen at an earlier (2 hours after exposure) time point [Mills *et al*, 2005]. We have now confirmed similar reductions in acute t-PA release 6 hours after diesel exhaust inhalation in patients with coronary heart disease. This delayed effect on endogenous fibrinolysis cannot explain our findings of immediate myocardial ischaemia but is consistent with the observations of Peters and colleagues who reported a second peak in the incidence of myocardial infarction 5-6 hours after traffic exposure [Peters *et*

al, 2004]. Pre-clinical thrombotic models also lend support to our findings. In a hamster model, Nemmar and colleagues have reported that instillation of diesel exhaust particulate into the lungs increases venous and arterial thrombus formation at sites of vascular injury [Nemmar *et al*, 2003a]. Taken together, these findings indicate an important thrombotic effect of diesel exhaust inhalation that may promote coronary thrombosis.

Although we found important adverse effects of diesel exhaust on vascular fibrinolytic function, we did not demonstrate an effect on vasomotor function. However, it is important to appreciate that vasomotor function was assessed 6 hours after exposure and 5 hours after we documented an increase in ischemic burden. We have previously demonstrated that exposure to diesel exhaust impairs vasomotor function in healthy volunteers [Mills *et al*, 2005]. This effect was most marked at 2 hours but was still present 6 hours after exposure. We cannot therefore exclude a detrimental vasomotor effect in patients at an earlier time point.

Patients with coronary heart disease have impaired endothelial function [Zeicher *et al*, 1991], and we have confirmed the presence of endothelial dysfunction in our study. This may have hindered our ability to demonstrate a further impairment of vascular function following diesel exhaust exposure. In addition, we performed our assessments in the presence of concomitant medications that are known to influence endothelial vasomotor function [Treasure *et al*, 1995]. Furthermore, Brook and colleagues have reported no effect of air pollution on endothelium-dependent vasodilatation [Brook *et al*, 2002].

We have identified two distinct and potentially synergistic adverse cardiovascular effects of air pollution in patients with coronary heart disease. These effects provide plausible mechanisms that may contribute to the increased incidence of myocardial infarction following exposure to traffic. However, the precise mechanisms whereby diesel exhaust inhalation induces these ischemic and thrombotic effects have not been established in our study and will need to be addressed in future work.

Our findings are consistent with recent epidemiological studies that report associations between ambient particulate air pollution and increased myocardial ischaemia on formal exercise testing [Gold *et al*, 2005; Pekkanen *et al*, 2002].

Myocardial ischaemia occurs as a consequence of reduced myocardial oxygen supply or increased demand or both. We hypothesize that oxidative stress and microvascular dysfunction in the resistance vessels of the myocardium may, in part, explain the adverse ischemic effects of exposure to dilute diesel exhaust. *In vitro* studies, animal models and human exposures have clearly established the oxidant and pro-inflammatory nature of combustion-derived particulate matter [Donaldson *et al*, 2003]. Indeed, the pattern of vascular dysfunction in our previous studies suggested a role for oxidative stress and reduced nitric oxide availability in mediating the adverse vascular effects of diesel exhaust inhalation [Mills *et al*, 2005].

Diesel exhaust is a complex mixture of gases and particles, and from our findings we cannot exclude a non-particulate cause of these adverse cardiovascular effects. However, in epidemiologic studies [Brunekreef and Holgate, 2002], particulate

matter has been held responsible for the majority of the adverse health effects of air pollution [Schwartz, 1994]. This view is supported by the recent observations of Miller *et al* in which they report a strong association between long-term exposure to particulate matter and cardiovascular outcome but no association with gaseous pollutants [Miller *et al*, 2007]. Ambient nitrogen dioxide can be considered a surrogate for traffic derived pollution, but has little adverse effect in controlled chamber studies even at the exposure levels seen here [Blomberg, 1997]. We therefore suggest that the cardiovascular effects described here are mediated primarily by diesel exhaust particulate and not its other components. This argues for the use of diesel exhaust particle traps to limit the adverse health impact of traffic emissions. However, the causative association of particulates must first be definitively established and the efficacy of particle traps confirmed.

Brief exposure to dilute diesel exhaust increases myocardial ischemia and impairs endogenous fibrinolytic capacity in men with stable coronary heart disease. Our findings suggest pathophysiologic mechanisms for the observation that exposure to combustion-derived air pollution is associated with adverse cardiovascular events including acute myocardial infarction. Environmental health policy interventions targeting reductions in urban air pollution should be considered in order to decrease the risk of adverse cardiovascular events.

CHAPTER 7

EXPOSURE TO CONCENTRATED AMBIENT PARTICULATE DOES NOT IMPAIR VASCULAR FUNCTION IN MAN

Published by Mills NL, Robinson SD, Fokkens P, Leseman D, Miller MR, Anderson D, Freney EJ, Heal MR, Donovan RJ, Blomberg A, Sandström T, MacNee W, Boon NA, Donaldson K, Newby DE, Cassee FR. Exposure to concentrated ambient particles does not affect vascular function in patients with coronary heart disease.

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7.1 SUMMARY

Exposure to fine particulate air pollution is associated with increased cardiovascular morbidity and mortality. We previously demonstrate that exposure to combustion-derived particulate from an idling diesel engine causes vascular dysfunction in man. In this study we wish to determine whether exposure to increased concentrations of 'real world' ambient particulate matter causes vascular dysfunction. Twelve male patients with stable coronary heart disease and 12 age-matched volunteers were exposed to fine + ultrafine CAPs or filtered air for 2 hours using a randomized double-blinded cross over study design. Bilateral forearm blood flow, fibrinolytic and inflammatory variables were measured 6-8 hours after CAPs or filtered air in response to intra-brachial agonist infusions. Particulate concentrations in the exposure chamber were higher than ambient levels and filtered air (190 ± 37 vs 31 ± 8 vs $0.5 \pm 0.4 \mu\text{g}/\text{m}^3$; $P < 0.001$). Chemical analysis of CAPs identified low levels of elemental carbon. Exhaled breath 8-isoprostane concentrations increased after exposure to CAPs (16.9 ± 8.5 pg/ml vs 4.9 ± 1.2 pg/ml, $P < 0.05$) but markers of systemic inflammation were largely unchanged. Although there was a dose dependent increase in blood flow with each vasodilator and plasma t-PA release ($P < 0.001$), this was unaffected by CAPs in either group. Despite achieving marked increases in particulate concentration, exposure to CAPs did not affect vasomotor or fibrinolytic function in either middle aged healthy volunteers or patients with coronary heart disease. Exposure to particulate air pollution low in combustion component is unlikely to mediate vascular effects capable of triggering an acute coronary events.

7.2 INTRODUCTION

Epidemiological and clinical research accumulated over 50 years has established the adverse effects of air pollution on human health. The London smog of December 1952 caused more than 4,000 excess deaths [Ministry of Health, 1954], and despite the dramatic decreases in levels of air pollution that have been achieved since then, the association between air pollution and cardiorespiratory morbidity and mortality persists [Anderson *et al*, 1996; Dockery *et al*, 1993; Pope *et al*, 2002]. These associations are strongest for PM_{2.5} and the majority of excess deaths are due to cardiovascular events [Pope, 2000]. Despite the strength of the epidemiological evidence and the emergence of promising hypotheses, the constituents and biological mechanisms responsible for the cardiovascular effects of air pollution are only beginning to emerge.

Exposure to particulate air pollution has been associated with exercise-induced myocardial ischaemia in patients with coronary heart disease [Pekkanen *et al*, 2002] and the triggering of acute myocardial infarction [Peters *et al*, 2001a]. These findings are limited by exposure misclassification, the effect of potential confounding environmental and social factors, and the lack of mechanistic data [Stone, 2004]. Controlled exposures of air pollutants can help to address these shortcomings by providing a precisely defined exposure in a regulated environment that facilitates investigation with validated biomarkers and surrogate measures of cardiovascular health.

Using a carefully characterised exposure system, we have previously shown that exposure to dilute diesel exhaust causes lung inflammation [Salvi *et al*, 1999], depletion of airway antioxidant defences [Behndig *et al*, 2006], and impairment of vascular and fibrinolytic function [Mills *et al*, 2005]. Whilst controlled exposure to diesel exhaust is an excellent model for studying the effects of pure combustion-derived air pollution we acknowledge that ambient air pollution contains a range of particulate pollutants from a variety of atmospheric sources. In the last few years, technology has been developed that can deliver a continuous flow of air in which the concentration of ambient particulate is increased in real-time [Sioutas *et al*, 1997, Sioutas *et al*, 1995]. The principle advantage of these concentrators is that they provide ‘real-world’ inhalation exposures under controlled conditions.

In previous studies, exposure to fine concentrated ambient particulate (CAPs) induced mild pulmonary inflammation in healthy adults [Ghio *et al*, 2000], and altered heart rate variability in the elderly [Devlin *et al*, 2003] and in asthmatic and healthy younger adults [Gong *et al*, 2003]. Furthermore, exposure to CAPs plus ozone causes peripheral arterial vasoconstriction [Brook *et al*, 2002] and an increase in arterial pressure [Urch *et al*, 2005]. To date no studies have addressed the effects of exposure to CAPs in isolation on vascular function, nor have any previous studies assessed the effect of these exposure in an ‘at risk’ population of patients with established coronary heart disease. We therefore assess the effects of a two hour exposure to fine + ultrafine CAPs on vascular function, fibrinolysis and inflammatory markers in patients with stable coronary heart disease and age-matched healthy controls.

7.3 METHODS

7.3.1 Subjects

Twelve male patients with stable coronary heart disease and 12 age-matched male non-smoking volunteers participated in these studies, which were performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and the written informed consent of all volunteers.

All patients had proven coronary heart disease with a previous myocardial infarction or stable angina treated by angioplasty and stenting (>6 months prior to enrolment), and were receiving standard secondary preventative therapy (Table 7.1). Patients with angina pectoris (Canadian Cardiovascular Society grade ≥ 2), a history of arrhythmia, diabetes mellitus, uncontrolled hypertension, renal or hepatic failure, or those with unstable coronary disease (acute coronary syndrome or unstable symptoms within 3 months) were excluded. Matched control subjects were not taking regular medication and had no clinical evidence of atherosclerotic vascular disease, diabetes mellitus, hypertension, renal or hepatic failure.

In both groups, current smokers and those with asthma, significant occupational exposure to air pollution, or an inter-current illness were excluded from the study. All subjects had normal lung function and none of them reported symptoms of respiratory tract infection during the study or in the preceding six weeks.

7.3.2 Study design

Subjects attended on two occasions two weeks apart and received fine + ultrafine CAPs or filtered air in a randomised double-blind cross-over design. Each subject was exposed for two hours in a specially built whole body exposure chamber. During each exposure subjects performed moderate exercise (minute ventilation 25 L/min/m²) on a bicycle ergometer that was alternated with rest at 15 minute intervals.

Based on previous exposure [Mills *et al*, 2005] and systemic inflammatory [Salvi *et al*, 1999] studies, vascular assessments were performed 6-8 hours following CAPs or filtered air exposure. All subjects abstained from alcohol for 24 hours and from food, tobacco and caffeine-containing drinks for at least 4 hours before each vascular study. Studies were carried out in a quiet, temperature controlled room maintained at 22-24°C with subjects lying supine. All subjects remained indoors between the exposure and vascular assessment to minimise additional exposure to particulate air pollution.

7.3.3 Concentrated ambient particle exposures and characterisation

A Versatile Aerosol Concentration Enrichment System (VACES) concentrator [Kim *et al*, 2000], within a Mobile Ambient Particle Concentrator Exposure Laboratory (MAPCEL) was used to deliver exposures to concentrated fine + ultrafine particles (CAPs, diameter <2.5 µm) and filtered air. The CAPs were derived from an urban background site outside the Royal Infirmary Edinburgh (Ordnance Survey Grid Reference, NT 289 703) approximately 6 miles from the centre of Edinburgh in Scotland, United Kingdom. A bus route passed adjacent to the MAPCEL and an

arterial city-route was located a few hundred meters away.

The air in the exposure chamber was continuously monitored for temperature, humidity, NO_x, CO, SO₂, and ozone. Particle number was determined using a condensation particle counter. Particle mass was continuously monitored using a DataRam nephelometer to standardise diesel exposures, with the precise mass determined by gravimetric filter measurements. The concentration of CAPs was not standardised for all subjects, as exposures were dependent on ambient particulate levels on the day of the study. We aimed to deliver approximately 200 µg/m³ to allow comparison with previously published studies [Mills *et al*, 2005].

An Aerosol Time-Of-Flight Mass Spectrometer (ATOFMS) was employed to characterise the single particulate matter sampled during the CAPS exposures. The operation of ATOFMS instrument has been described in detail elsewhere [Toner *et al*, 2006] and detailed results are presented elsewhere [Freney *et al*, 2006].

7.3.4 Vascular studies

All subjects underwent brachial artery cannulation with a 27-gauge steel needle under controlled conditions. Following a 30 min baseline saline infusion, acetylcholine at 5, 10 and 20 µg/min (endothelium-dependent vasodilator that does not release t-PA; Merck Biosciences, Switzerland), bradykinin at 100, 300 and 1000 pmol/min (endothelium-dependent vasodilator that releases t-PA; Merck Biosciences, Switzerland) and sodium nitroprusside at 2, 4 and 8 µg/min (endothelium-independent vasodilator that does not release t-PA; David Bull

Laboratories, UK) were infused for 6 min at each dose. The three vasodilators were separated by 20 min saline infusions and given in a randomised order. Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges. Supine heart rate and blood pressure in the non-infused arm were monitored at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer.

7.3.5 Fibrinolytic and Inflammatory Markers

Venous cannulae (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood (10 mL) was withdrawn simultaneously from each arm at baseline and during the infusion of each dose of bradykinin, and collected into acidified buffered citrate (Stabilyte tubes, Biopool International) for t-PA assays, and citrate (BD Vacutainer) for PAI-1 assays. Samples were kept on ice before being centrifuged at 2000 g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA and PAI-1 antigen concentrations were determined by enzyme linked immunosorbant assays (TintElize t-PA, Biopool EIA; Coaliza PAI-1, Chromogenix AB). Haematocrit was determined by capillary tube centrifugation at baseline and during infusion of bradykinin 1000 pmol/min.

Blood samples were taken immediately before, 6 and 24 hours after the exposure and analysed for total cells, differential count and platelets using an autoanalyzer. Serum CRP concentrations were measured using an immunonephelometric assay (Behring BN II nephelometer, Marburg, Germany).

7.3.6 Exhaled breath condensate

In a pilot study exhaled breath condensate (EBC) was collected using a Jaeger Ecoscreen (VIASYS Healthcare, Hoechberg, Germany) immediately before, 6 and 24 hours after exposures in 8 healthy volunteers. Subjects rinsed their mouths with water immediately prior to the collection. EBC was collected during a 10-minute period of normal tidal breathing through the mouthpiece with a nose clip in place. Samples were placed on ice immediately after collection and aliquoted for storage at -80°C within 30 minutes. 8-isoprostane and nitrotyrosine were measured in EBC using commercially available enzyme-linked immunosorbent assays (Quantikine, R&D Systems, Minneapolis).

7.3.7 Data analysis and statistics

Plethysmographic data were analysed as described previously [Newby *et al*, 1999]. Estimated net release of t-PA antigen was defined as the product of the infused forearm plasma flow (based on the mean haematocrit and the infused forearm blood flow) and the concentration difference between the infused and non-infused arms [Newby *et al*, 1997]. Continuous variables are reported as mean \pm SEM. Statistical analyses were performed with GraphPad Prism (Graph Pad Software) using ANOVA with repeated measures, Pearson's correlation, and two-tailed Student's *t*-test where appropriate. The area under the curve was calculated for the estimated net release of t-PA during the forearm study period. Statistical significance was taken at $P < 0.05$.

7.4 RESULTS

Subjects tolerated the exposures well and did not report any symptoms during or in the 24-hours after each exposure. Patients and controls were well matched for age and blood pressure (Table 7.1), and all patients were on optimal medication for secondary prevention. Patients with coronary artery disease were shorter and more overweight with greater BMI than the healthy controls ($P<0.05$).

Table 7.1 Baseline characteristics of coronary heart disease patients and controls

	Controls	Patients	Significance
Age (years)	54±2	59±2	0.12
Height (cm)	181±1	176±2	0.03
Weight (kg)	81±3	87±3	0.14
Body mass index (m ² /kg)	25±1	28±1	0.02
FEV1 (L)	3.4±0.1	3.0±0.2	0.04
FVC (L)	4.6±0.2	4.2±0.2	0.07
Ratio	74±1	72±2	0.32
Systolic blood pressure (mmHg)	135±5	138±6	0.69
Diastolic blood pressure (mmHg)	77±3	80±3	0.50
Heart rate (bpm)	63±3	54±1	0.006
Previous MI	0	7	
Previous PTCA	0	7	
Previous CABG	0	1	
Current-smokers	0	0	

Ex-smokers (Non-/Ex-/Current)	3	5
Non-smokers (Non-/Ex-/Current)	9	7
Hypertension	0	4
Hypercholesterolaemia	0	12
Drugs		
Aspirin	0	12
Statin	0	12
B-blocker	0	11
ACE inhibitor*/ARB	0	7

Values are presented as number or mean \pm SEM or number;

FEV= forced expiratory volume in 1 second;

FVC= forced vital capacity;

MI = myocardial infarction;

PTCA = percutaneous transluminal coronary arteriography;

CABG = coronary artery bypass grafting;

ACE = angiotensin converting enzyme;

ARB = angiotensin receptor blocker

*ACE inhibitor therapy was withdrawn 7 days prior to each vascular study. All other regular medications were continued throughout the study.

7.4.1 Exposures to ambient particles

Ambient particulate concentrations were variable (Range 3-174 $\mu\text{g}/\text{m}^3$) during the three month duration of the study with an average concentration of $20\pm 4 \mu\text{g}/\text{m}^3$.

Particulate concentrations in the exposure chamber were higher than ambient levels and filtered air (190 ± 37 vs 31 ± 8 vs $0.5\pm 0.4 \mu\text{g}/\text{m}^3$; $P<0.001$). There were no significant differences between concentrations of gaseous co-pollutants between the filtered air or CAPs exposures (Table 7.2). There were however differences in humidity and temperature in the chamber between the CAPs and filtered air exposures that occurred as a result of the saturation and dilution phases of the enrichment process.

Table 7.2 Exposure variables

	<i>Air</i>	<i>CAPs</i>	<i>Significance</i>
PM [Teflon filter] ($\mu\text{g}/\text{m}^3$)	-	178 \pm 46	<0.001
PM [Quartz filter] ($\mu\text{g}/\text{m}^3$)	-	162 \pm 22	<0.001
PM [Data Ram] ($\mu\text{g}/\text{m}^3$)	0.5 \pm 0.4	190 \pm 37	<0.001
Ambient PM [Data Ram] ($\mu\text{g}/\text{m}^3$)	9 \pm 1	31 \pm 8	0.01
Particle no. (1,000 x number/ cm^3)	-	99.4 \pm 9.5	<0.001
Ozone (ppb)	6.0 \pm 1.3	5.0 \pm 1.2	0.52
Carbon monoxide (ppb)	27 \pm 1	24 \pm 3	0.40
Sulphur dioxide (ppb)	0.13 \pm 0.07	0.13 \pm 0.07	1.00
Nitric oxide [NO] (ppb)	4.5 \pm 0.3	4.6 \pm 1.0	0.93
Nitrogen dioxide [NO ₂] (ppb)	5.9 \pm 0.7	5.1 \pm 1.0	0.52
NO _x [NO +NO ₂] (ppb)	6.3 \pm 0.7	7.2 \pm 1.7	0.60
Temperature ($^{\circ}\text{C}$)	21.1 \pm 0.2	20.1 \pm 0.2	0.004
Relative humidity (%)	28 \pm 2	57 \pm 2	<0.001

Values are presented as number or mean \pm SEM (n=24)

Using the ATOFMS, elemental analysis of ambient particulate was acquired during each two-hour exposure for a representative two-week period of the study. The ATOFMS detected low levels of carbon ($5.3\pm1.2\%$ of all analysed particles), and sodium chloride ($91.7\pm6.5\%$) as the primary chemical constituent of ambient aerosol (Figure 7.1).

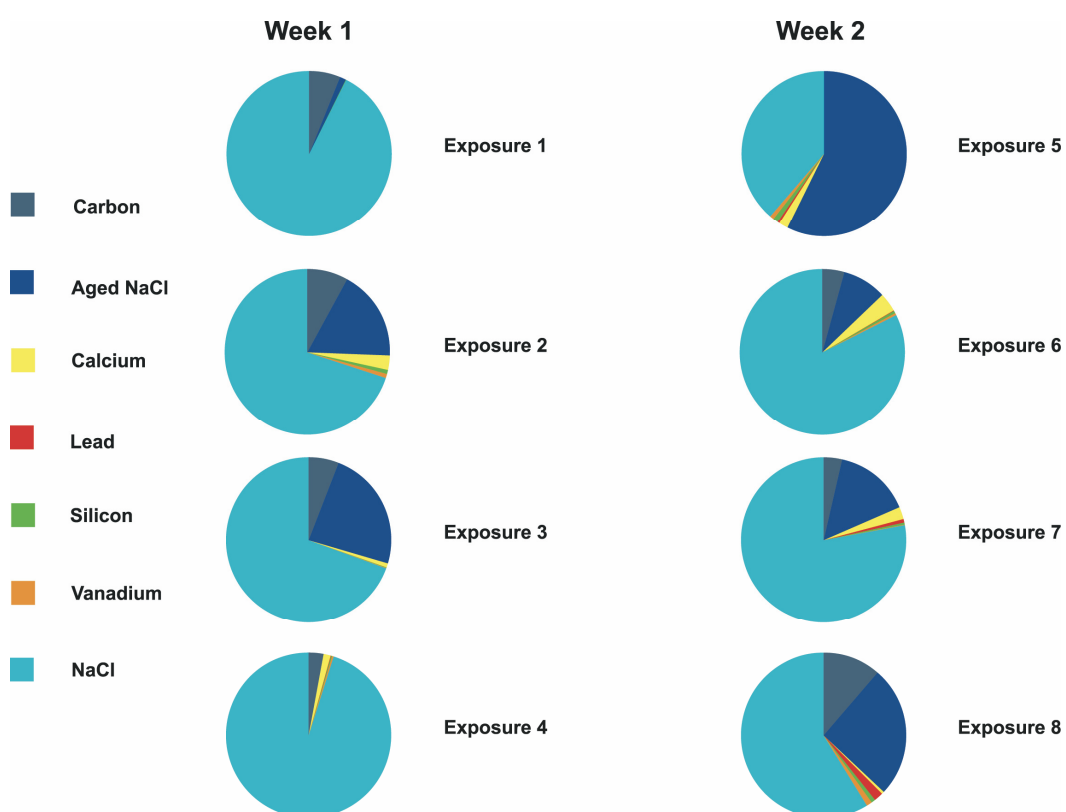


Figure 7.1 ATOFMS analysis of ambient particulate during each two-hour exposure in a representative two-week period. The ATOFMS detected low levels of carbon, and sodium chloride as the primary chemical constituent of ambient aerosol.

Vascular function

There were no differences in resting heart rate, blood pressure or baseline forearm blood flow following exposure to CAPs or air in either cohort (Table 7.3).

Bradykinin, acetylcholine, and sodium nitroprusside caused dose-dependent increases in forearm blood flow following both air and CAPs exposure ($P < 0.0001$), however this increase in blood flow was not affected by exposure to CAPs or filtered air in either patients or controls (Figure 7.2). Bradykinin caused a dose-dependent increase in plasma t-PA antigen concentrations ($P < 0.0001$) that was similarly unaffected by exposure (Table 7.4). We found no correlation between peak forearm blood flow and particle mass or particle number in the CAPs exposure for any of the vasodilators infused.

Table 7.3 Haemodynamics variables 6 hours following exposure in all subjects

	<i>Air</i>	<i>CAPs</i>	<i>Significance</i>
Heart rate (bpm)	58±2	58±1	0.94
Systolic blood pressure (mmHg)	135±4	135±4	0.51
Diastolic blood pressure (mmHg)	78±2	78±2	0.96
Mean arterial pressure (mmHg)	97±2	97±3	0.70
Infused FBF (mL/100 mL tissue/min)	2.6±0.3	2.5±0.2	0.57
Non-infused FBF (mL/100 mL tissue/min)	2.7±0.2	2.3±0.2	0.16

Values are reported as mean ± SEM (n=24), two-tailed paired *t*-test

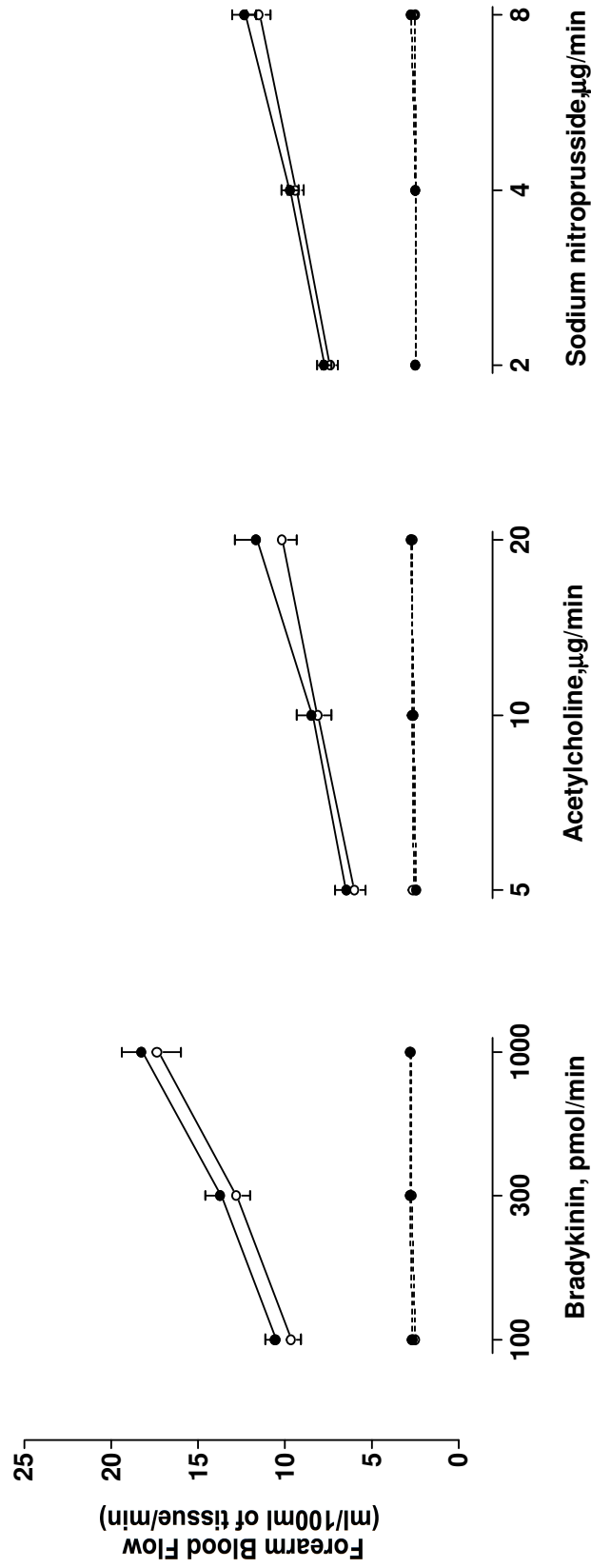


Figure 7.2 Infused (solid line) and non-infused (dashed line) forearm blood flow in all subjects following CAPs (●) and filtered air (○) exposures during intra-brachial infusion of bradykinin, acetylcholine and sodium nitroprusside: for all dose responses in the infused arm $P < 0.0001$. For CAPs (●) versus filtered air (○); bradykinin ($P = 0.20$), acetylcholine ($P = 0.17$) and sodium nitroprusside ($P = 0.14$).

Table 7.4 Plasma t-PA antigen concentrations following filtered air and concentrated ambient particles

	Air				CAPs			
	0	100	300	1000	0	100	300	1000
Bradykinin, pmol/min								
tPA antigen, ng/mL								
Non-infused arm	7.6±0.5	7.4±0.5	7.9±0.5	8.5±0.5	7.2±0.5	7.3±0.6	7.7±0.5	8.6±0.5
Infused arm	7.3±0.5	8.8±0.5	9.7±0.6	14.3±1.2*	7.3±0.6	8.6±0.6	9.5±0.7	14.2±1.1*
Difference	-0.4±0.2	1.4±0.4	1.9±0.4	5.8±1.1*	0.1±0.2	1.3±0.4	1.7±0.4	5.7±1.0*
Net t-PA release, ng/100ml of tissue/min	-1±1	11±3	20±5	90±18*	0±1	12±4	24±6	104±19*

Values are reported as mean ± SEM; ANOVA (dose response), *P<0.0001

Markers of oxidative stress and inflammation

Exhaled breath condensate levels of 8-isoprostane increased 6- and 24-hours after CAPs exposure compared to filtered air (ANOVA, $P=0.04$; Figure 7.3) where as levels of nitrotyrosine did not significantly differ between exposures. In all subjects there was a small increase in the number of circulating platelets (234 ± 8 vs. $225 \pm 8 \times 10^9/L$, $P=0.007$ at 2-hours) and monocytes (0.58 ± 0.03 vs. $0.53 \pm 0.03 \times 10^9/L$, $P=0.03$ at 6-hours) following exposure to CAPs versus filtered air (Table 7.5). There was however no clear evidence of a systemic inflammatory response to CAPs exposure with total leucocyte, neutrophil, and lymphocyte counts or serum CRP concentrations unaltered by CAPs or air exposure at any time point (Table 7.5).

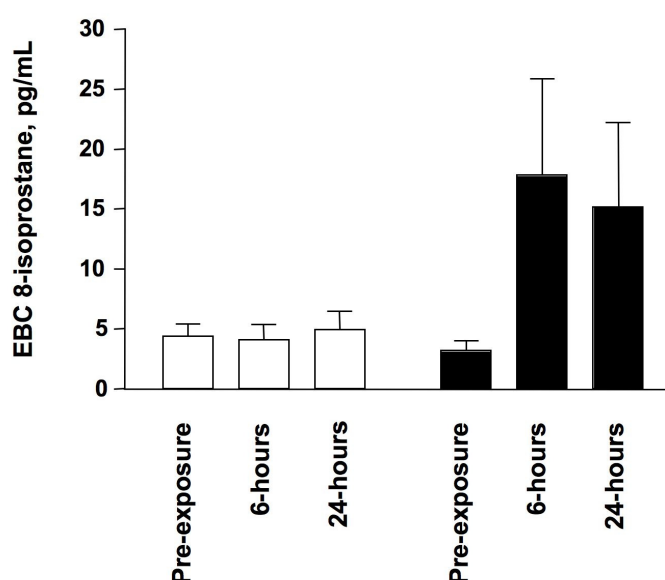


Figure 7.3 In health volunteers ($n=8$) exhaled breath condensate levels of 8-isoprostane increased 6- and 24-hours after CAPs (●) compared to filtered air (○) (ANOVA, $P=0.04$).

Table 7.5 Systemic effects of exposure to filtered air or concentrated ambient particles (CAPs)

	Air					CAPs			
	Pre	2-hrs	6-hrs	24-hrs	Pre	2-hrs	6-hrs	24-hrs	ANOVA
Leucocytes (x 10 ⁹ cells/L)	5.8±0.2	5.8±0.2	6.4±0.2	5.6±0.2	5.8±0.2	6.1±0.2	6.4±0.2	5.5±0.2	0.31
Neutrophils (x 10 ⁹ cells/L)	3.2±0.1	3.5±0.1	3.9±0.2	3.3±0.1	3.3±0.1	3.7±0.2	3.9±0.2	3.2±0.2	0.58
Lymphocytes (x 10 ⁹ cells/L)	1.7±0.1	1.5±0.1	1.7±0.1	1.5±0.2	1.6±0.1	1.6±0.1	1.8±0.1	1.5±0.1	0.47
Monocytes (x 10 ⁹ cells/L)	0.57±0.03	0.50±0.03	0.53±0.03	0.49±0.03	0.55±0.04	0.55±0.04	0.58±0.04*	0.50±0.03	0.02
Platelets (x 10 ⁹ cells/L)	221±7	225±8	218±7	223±7	224±8	234±8*	217±6	228±8	0.04
CRP (mg/L)	1.2±0.2	-	1.1±0.2	1.3±0.2	1.3±0.2	-	1.2±0.2	1.2±0.2	0.72
PAI-1 antigen (ng/mL)	57±5	33±3	19±2	48±5	63±5	34±3	19±2	51±4	0.30
t-PA antigen (ng/mL)	10.1±0.6	10.6±0.7	7.2±0.5	10.6±0.7	10.2±0.8	10.5±0.7	7.2±0.5	10.9±0.7	0.86

Values are reported as mean ± SEM (n=24)

Repeated measure ANOVA CAPs *versus* filtered air

Student's *t*-test CAPs *versus* filtered air (time point): * P<0.05

7.5 DISCUSSION

Exposure to concentrated ambient particulate for two hours in a typical urban environment did not affect vascular vasomotor or endogenous fibrinolytic function in either middle-aged healthy volunteers or patients with coronary heart disease. Edinburgh ambient particulate was generally low in elemental carbon indicating that combustion sources were not a major source of ambient particulate. Inhalation of CAPs caused mild pulmonary oxidative stress, but did not result in a significant systemic inflammatory response. Exposure to particulate air pollution low in combustion component, even at concentrations 5-fold higher than the United States Environmental Protection Agency (EPA) National Ambient Air Quality Standards (NAAQS), is unlikely to cause significant adverse vascular effects capable of triggering an acute coronary events.

These findings contrast those from our previous studies in which we report impairments of vascular function in both healthy volunteers patients with coronary heart disease following a one hour exposure to dilute diesel exhaust [Mills *et al*, 2005; Törnqvist *et al*, 2007]. This apparent discrepancy requires further discussion with differences in particle number, particle composition, and the presence of gaseous co-pollutants between these exposures potentially responsible.

7.5.1 Ambient particle and diesel exhaust exposures

We were able to increase the concentration of ambient particulate 6- to 8-fold to deliver exposures of between 50 and 682 $\mu\text{g}/\text{m}^3$. The majority of exposures were substantially higher than the USEPA standard of 65 $\mu\text{g}/\text{m}^3$ for daily $\text{PM}_{2.5}$. Exposure to 190 $\mu\text{g}/\text{m}^3$ for 2 hours is roughly comparable to a one-hour exposure to the 300 $\mu\text{g}/\text{m}^3$ of diesel exhaust particulate delivered in previous studies. We are confident that these on-line estimates of exposure are accurate as the precise mass determined by gravimetric filter measurements. It is possible that vascular dysfunction only occurred in subjects exposed to the highest concentrations of CAPs and that a significant overall effect of exposure to CAPs on vascular function was masked by those volunteers receiving only modest particle exposures. This seems unlikely given we found no relationship between particle number or mass and vasodilatation to any of the endothelial-dependent or -independent agonists infused.

Whilst we delivered a similar mass of particulate in our present study and previous exposures to diesel exhaust, there were important differences in particle size and therefore particle number concentration between the exposures. In dilute diesel exhaust the particulate consists of pure combustion-derived carbon nanoparticulate. These particles typically range from 20 to 120 nm, with a count median diameter of 54 nm (geometric standard deviation = 1.7 nm) [Mills *et al.* 2007]. The mean particle size in a typical CAPs exposure was $1.2 \pm 0.4 \mu\text{m}$ in diameter. The total number of suspended particles in dilute diesel exhaust was therefore 10-fold more than in the CAPs exposure. Toxicological studies have determined that the adverse oxidative and pro-inflammatory effects of particles are in part determined by surface area

[Duffin *et al*, 2007]. It follows that smaller particles exert a greater effect per unit mass than larger particles of similar toxicity. It is therefore possible that the lack of adverse vascular effects following exposure to CAPs reflects the relatively low number concentration of particles in the present study.

Diesel exhaust is a complex mixture of gases and particles, and whilst we hypothesise that our previous findings are principally due to an effect of combustion-derived particles, it is not possible to definitively exclude a non-particulate cause of the adverse vascular effects. Potentially important co-pollutants produced in the combustion of diesel oil include nitrogen oxides and carbon monoxide. One of the advantages of the VACES system is that it allows us to study the effects of particulates alone and the enrichment process does not alter the concentration of gaseous pollutants. Concentrations of NO₂ and CO were low and easily within the recommended EPA's NAAQS in our present study. Whilst in epidemiological studies ambient NO₂ or CO have been primarily considered surrogates for traffic derived pollution [Brunekreef and Holgate, 2002], it is possible that these gaseous pollutants in diesel exhaust exert a synergistic effect with the particulates. There are no previous studies addressing the effects of NO₂ or CO in isolation on the vascular or fibrinolytic systems, however controlled chamber studies suggest that NO₂ even at the exposure levels seen here does not cause significant lung inflammation [Blomberg *et al*, 1997].

As an important source of combustion-derived particulate, diesel exhaust is strongly implicated in the observed adverse effects of air pollution. A variable proportion of

urban PM is attributable to combustion-derived nanoparticles from traffic; ranging from 20% at remote monitoring sites [Lanki *et al*, 2006] up to 70% in a road tunnel [Geller *et al*, 2005]. In Edinburgh CAPs, only 5.3% of particles analysed contained elemental carbon and the principle constituent was pure, mixed or reacted sea-salts (91.7 ± 6.5 %). This is not surprising given Edinburgh's maritime climate. Air-mass source attribution plots for the 5 days prior to arrival at the sampling location were calculated for each of the exposure periods using a United Kingdom Met Office model [Manning AJ, 2003]. In all cases air clearly originated either predominantly from the Atlantic or the Arctic with very little contribution from air passing over land apart from final arrival over central Scotland [Freney *et al*, 2006]. The proportion of airborne particulate derived from remote combustion sources is likely to be low. It is very likely that the absence of any detrimental vascular effects in the present study in part reflect the composition of Edinburgh CAPs, which is likely to be very low toxicity.

7.5.2 Air pollution, oxidative stress and inflammation

A substantial body of evidence supports a role for oxidative stress in determining the toxicity of ambient pollution [Donaldson *et al*, 2005a] and in the pro-inflammatory effects of combustion-derived particles [Donaldson *et al*, 2005b; Nemmar *et al*, 2003b]. Reactive oxidant species arise not only from the redox potential of the pollutants themselves, but also from the activation of alveolar epithelial cells or resident macrophage and the recruitment of circulating neutrophils. In our study we used an emerging non-invasive method of assessing pulmonary oxidative stress through collection of exhaled breath condensate [Montuschi *et al*, 1999]. In these

preliminary studies we found an increase in exhaled breath 8-isoprostane suggesting that inhaled ambient particles exert a pro-oxidant effect in the airways. This is the first time to our knowledge this technique has been used to assess the effects of exposure to air pollutants and if these findings can be replicated and extended in a larger study breath condensate measures may become a useful biomarker of PM exposure.

In panel and population studies PM exposure is associated with evidence of an acute phase response with increased CRP [Peters *et al*, 2001b] and plasma fibrinogen [Pekkanen *et al*, 2000; Schwartz, 2001], enhanced plasma viscosity [Peters *et al*, 1997] and altered haematological indices [Seaton *et al*, 1999]. In animal studies there are similar reports with increased fibrinogen in the blood of PM-exposed hypertensive rats [Cassee *et al*, 2002] and normal rats exposed to ultrafine carbon particles [Elder *et al*, 2004b]. We did not find a consistent systemic inflammatory signal with no change in the number of circulating neutrophils, lymphocytes, or total leucocytes even up to 24 hours following CAPs exposure. Similarly we did not find an increase in serum CRP concentrations suggesting that a 2-hour exposure to CAPs is not sufficient to cause a sustained systemic inflammatory response.

The number of circulating platelets increased 2 hours following CAPs exposure and a similar transient effect on circulating monocytes was present at 6 hours. Whether these small changes are likely to increase cardiovascular risk is questionable. Whilst the cellular mechanisms of atherosclerosis are complex, adhesion of platelets and monocytes to the damaged arterial wall occurs early in response to vascular injury

[Fuster *et al*, 1992]. Activated platelets deposit at sites of plaque rupture and may precipitate coronary artery occlusion. Platelet-monocyte aggregates are increased in cigarette smokers [Harding *et al*, 2004] and patients with unstable angina [Sarma *et al*, 2002] suggesting that leukocyte–platelet interactions may contribute to atheromatous plaque instability. Whilst we have not measured markers of platelet or monocyte activation in our study, it has recently been reported that inhalation of carbon ultrafine particles alters leukocyte expression of adhesion molecules in peripheral blood [Frampton *et al*, 2006].

7.5.3 Study limitations

Using a robust randomised double blind sham-exposure study design we have assessed the effect of CAPs on two complementary aspects of vascular function. Impaired vasodilatation and fibrinolytic function in the forearm vascular bed have previously been shown to independently predict adverse outcomes in patients with coronary heart disease [Heitzer *et al*, 2001; Robinson *et al*, 2007] and therefore we believe this model is a reasonable surrogate for cardiovascular health. Furthermore, these studies were conducted in a highly relevant population of patients with existing coronary heart disease who are likely to be susceptible to the adverse effects of air pollution and for whom a better understanding of the effects of exposure to particulate air pollution is of clinical importance. The findings are clear; a two-hour exposure to increased concentrations of ambient particulate in an urban setting does not have significant effects on systemic vascular function.

However, it is not possible to generalise our findings or state that in other urban settings exposure to PM is not likely to exert harmful vascular effects. This is primarily because the maritime climate and location of the MAPCEL resulted in an exposure to low levels of combustion component. The concentrator technology relies on environmental conditions on the study date to ensure that a relevant exposure can be delivered, and therefore we were not able to give a pre-specified concentration of ambient particulate. Whilst we delivered almost $200 \mu\text{g}/\text{m}^3$ it is possible that higher concentrations even of low toxicity CAPs would have had a more potent adverse effects. Further studies in different cities and city locations are clearly warranted.

Conclusions

Despite achieving substantial increases in ambient particulate concentrations in an urban setting exposure to ambient particulate air pollution for two hours had no effect on vascular vasomotor or endogenous fibrinolytic function in either healthy middle-aged volunteers or patients with established coronary heart disease. These findings suggest that exposure to particulate matter that is low in combustion component is unlikely to exert significant vascular effects capable of triggering an acute coronary events.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

8.1 SUMMARY OF THESIS FINDINGS

Short and long-term exposure to air pollution is associated with increases in cardiovascular morbidity and mortality with deaths due to ischaemia, arrhythmia and heart failure [Brook *et al.*, 2004]. These associations are strongest for fine particulate air pollutants, of which combustion-derived particulate in diesel exhaust is an important component. Whilst the mechanisms responsible for these associations are only partly understood, observational studies suggest that exposure to air pollution may worsen symptoms of angina, exacerbate exercise induced myocardial ischemia, and trigger acute myocardial infarction [Peters *et al.*, 2006]. These clinical findings are limited by imprecision in the measurement of pollution exposure, the effect of potential confounding environmental and social factors, and the lack of mechanistic data. Controlled exposures of air pollutants can help to address these shortcomings by providing a precisely defined exposure in a regulated environment that facilitates investigation with validated biomarkers and surrogate measures of cardiovascular health.

In health the vascular endothelium delicately balances regulatory pathways controlling blood flow, coagulation, fibrinolysis and inflammation. It is widely recognized that a variety of risk factors including cigarette smoking can influence vascular tone through endothelium-dependent actions, and there is now extensive evidence of abnormal endothelium-dependent vasomotion in patients with atherosclerosis [Newby *et al.*, 1999 and 2001]. As combustion products and particulate matter are common to both air pollution and cigarette smoke, we

hypothesised that air pollution is likely to have similar detrimental effects on vascular function.

8.1.1 Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis

In a double-blind randomised crossover study, 30 healthy men were exposed to dilute diesel exhaust (300 $\mu\text{g}/\text{m}^3$ particulate concentration) or air for one hour during intermittent exercise. Using a powerful study design, we have assessed two important and complementary aspects of vascular function: the regulation of vascular tone and endogenous fibrinolysis. Both are impaired and plausibly related to the well-documented cardiovascular effects of air pollution [Mills *et al.*, 2005].

8.1.2 Persistent endothelial dysfunction after diesel exhaust inhalation

In our subsequent studies we demonstrate that endothelial dysfunction persists 24-hours following exposure and is associated with an increase in systemic inflammatory cytokines [Törnqvist *et al.*, 2007]. These changes in vascular function are less marked than seen at the earlier time points and the time-course of this vascular effect of exposure is more in keeping with enhanced oxidative stress. In support of this we demonstrate using electron paramagnetic resonance studies that diesel exhaust particles are capable of generating reactive oxidative species, and that this effect can be quenched by superoxide dismutase.

8.1.3 Do inhaled carbon nanoparticles translocate directly into the circulation in man?

Whether inhaled nanoparticles can cause vascular oxidative stress directly through translocation from the lungs into the circulation requires further study [Mills *et al.*, 2006]. In an attempt to extend the findings from the only previously published clinical study to address whether inhaled nanoparticles translocate we identify serious flaws in the original study. It seems likely that small quantities of inhaled particulate can reach the systemic circulation, however it is more plausible that the acute vascular effects are mediated by a soluble factor or a pro-inflammatory or oxidised protein released from the lungs.

8.1.4 Ischemic and thrombotic effects of dilute diesel exhaust inhalation in men with coronary heart disease

Recently the AHA published a scientific statement in which, on the strength of the observational studies, they advise patients to restrict physical activity on days in which air pollution levels are increased [Brook *et al.*, 2004]. Perhaps a surprisingly strong statement given there have been no controlled exposures to air pollutants in patients with coronary heart disease. Whilst the recent COMEAP report to the UK Department of Health acknowledges the role of air pollution in the pathogenesis of cardiovascular disease the authors of this report did not feel that there was sufficient evidence to support avoidance strategies.

Patients with coronary artery disease are an important population who may be particularly susceptible to the adverse cardiovascular effects of air pollution. We

have therefore assessed the effect of dilute diesel exhaust inhalation on myocardial, vascular and fibrinolytic function in an “at risk” population of patients with stable coronary heart disease. Twenty patients with prior myocardial infarction were exposed to dilute diesel exhaust ($300 \mu\text{g}/\text{m}^3$) or filtered air during periods of rest and moderate exercise in a controlled exposure facility. During the exposure, myocardial ischemia was quantified by ST-segment analysis using continuous 12-lead electrocardiography. Exercise induced ST-segment depression was present in all patients but there was a 3-fold greater increase in ST segment depression and ischemic burden during exposure to diesel exhaust. Exposure to diesel exhaust did not aggravate pre-existing vasomotor dysfunction, but did reduce acute endothelial t-PA release. We have demonstrated that transient exposure to dilute diesel exhaust, at concentrations occurring in urban road traffic, exacerbates exercise-induced myocardial ischemia and impairs endogenous fibrinolytic capacity in patients with coronary heart disease [Mills *et al.*, 2007].

8.1.5 Exposure to concentrated ambient particles does not affect vascular function in men with coronary heart disease

Finally we extend our studies to address whether concentrated real world particles exert similar detrimental effects on vascular function in both healthy volunteers and patients with coronary heart disease. These studies were conducted outside the Royal Infirmary in Edinburgh. Despite achieving a 10-fold increase in ambient fine particulate concentrations we did not find that a 2-hour exposure to Edinburgh ambient particulate caused any detrimental effects on either vasomotor or endogenous fibrinolytic function in patients or control subjects. The component of

ambient particulate arising from combustion processes was exceedingly low and we conclude that particle composition is critical in determining the adverse vascular effects of PM air pollution.

8.2 FUTURE DIRECTIONS

A number of questions have arisen from the results and peer review of the above studies that we hope to explore in future studies. There are several highly relevant clinical and environmental health issues that need to be addressed.

8.2.1 Are combustion-derived nanoparticles responsible for the adverse vascular effects of diesel exhaust?

Diesel exhaust is a complex mixture of gases, particles and volatiles, and our earlier studies preclude identification of the component(s) responsible for the adverse effects. Nitrogen dioxide, carbon monoxide, ozone, metals and organics (unburnt fuel and lube oil), and formaldehyde may play a role. We propose to identify the precise component of diesel exhaust that mediates the adverse cardiovascular effects using combinations of carbon particle generators, particle filtration, and pure gaseous exposures. This is an important consideration in the science of emission control technology, where the focus is on reducing the most important emission to gain a health advantage. A focused regulatory approach with the reduction of specific pollutants is likely to be the most cost effective approach to improving public health [Ware *et al*, 2000].

To address this question we will recruit sixteen healthy non-smoking volunteers who will attend on four occasions, more than two weeks apart, and receive dilute diesel exhaust ($300 \mu\text{g}/\text{m}^3$), filtered diesel exhaust (gaseous pollutants only), filtered air or ultrafine carbon particle exposures in a double-blind randomised cross-over fashion. They will perform moderate exercise or rest alternated at 15-minute intervals while sitting on a recumbent bicycle ergometer during each two-hour exposure. Forearm vascular responses and inflammatory markers will be determined 6-8 hours after the completion of each exposure.

All subjects will be exposed in the MAPCEL with modifications to allow controlled exposures to diesel exhaust, filtered diesel exhaust and carbon particles (Figure 8.1). The diesel exhaust will be generated from an unloaded diesel engine (Deutz, 4 cylinder, 2.2 L, 500 rpm) using standard commercially available gas oil (Petroplus Refining Teesside Ltd., United Kingdom). High efficiency particle filtration will be achieved by passing the dilute diesel exhaust through an impactor with a cutoff of $0.1 \mu\text{m}$ and a TE38 Teflon filter (Schleicher & Schuell, Dassel, Germany). Ultrafine 'pure' carbon particles will be generated from graphite electrodes in an electric spark discharge generator (Palas Soot Generator, Karlsruhe, Germany).

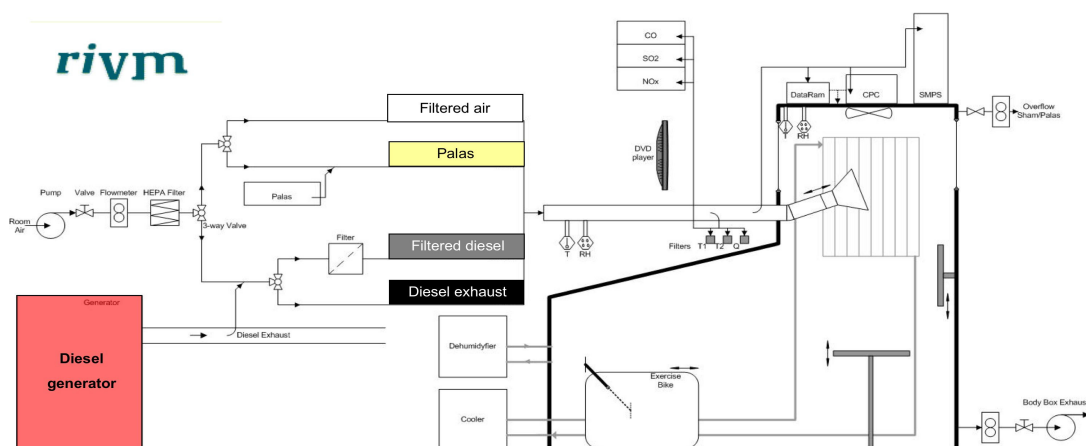


Figure 8.1 Diesel exhaust will be generated from an unloaded diesel engine using gas oil. More than 90% of the exhaust will be shunted away, and the remaining part diluted with air and fed at 75 L/min into the exposure chamber at steady-state concentration. Diesel exhaust particulate will be removed for the control filtered exhaust exposure by passing dilute exhaust through a Teflon filter. Carbon nanoparticles were generated using a spark discharge generator (Palas Soot Generator).

8.2.2 Are the adverse vascular effects of diesel exhaust related to the magnitude of exposure?

Particulate matter concentrations can regularly reach levels of $300 \mu\text{g}/\text{m}^3$ in heavy traffic, occupational settings, and in the world's largest cities [WHO, 1994]. A major proportion of this mass is attributable to combustion-derived nanoparticles from traffic; ranging from 20% at remote monitoring sites [Lanki *et al*, 2006] up to 70% in a road tunnel [Geller *et al*, 2005]. Exposure to $300 \mu\text{g}/\text{m}^3$ for one hour increases a person's average exposure over a 24-hour period by only $12 \mu\text{g}/\text{m}^3$. Changes of this magnitude occur on a daily basis, and are associated with increases in cardiorespiratory mortality [Dockery *et al*, 1993]. However, we recognise that many people may not be exposed to this level of air pollution. We therefore need to explore the dose-response relationship of these effects and determine if a threshold exists.

Sixteen non-smoking volunteers with normal lung function and no history of respiratory disease will be invited to attend on four separate days at least two weeks apart and receive a double-blind randomised cross-over exposure to filtered clean air or diesel exhaust at 30, 100 and $300 \mu\text{g}/\text{m}^3$. Vascular function will be determined 6-8 hours following the end of each exposure using venous occlusion plethysmography.

8.2.3 Does diesel exhaust inhalation reduces exercise capacity and increase ischaemic burden in patients with stable angina pectoris?

We have undertaken the first controlled diesel exhaust exposures in patients with coronary heart disease. Given potential safety concerns, we recruited stable asymptomatic patients who had been revascularized and had good exercise tolerance

on formal stress testing. Volunteers were closely monitored throughout the exposure and reported no adverse effects. Despite similar changes in heart rate, we documented painless myocardial ischaemia that was increased up to three-fold by diesel exhaust inhalation. This reproducible effect was present despite a high use of maintenance β -blocker therapy in patients without limiting angina. It seems likely that diesel exhaust inhalation will have greater impact in other patient populations, such as those with limiting angina. We therefore need to explore whether diesel exhaust inhalation has functional consequences including reduced exercise capacity in patients with stable angina pectoris. We also need to explore whether diesel exhaust inhalation has other cardiovascular consequences in such patients.

Twenty patients with stable angina pectoris will attend on four occasions at least two weeks apart. All subjects will have a 12-lead Holter monitor for the 24 hours during and following exposure. Subjects will be exposed to filtered air, 30, 100 and 300 $\mu\text{g}/\text{m}^3$. Exercise tolerance testing will be performed according to the Bruce protocol 2 hours following the start of each exposure, with the time to symptom onset and time to 1mm ST-segment depression recorded. Blood samples will be immediately before and after the exposure and the stress test, and at 24 hours to measure markers of systemic inflammation and myocardial ischaemia including high sensitive troponin, ischaemically modified albumin, fatty acid binding protein and NT-brain natriuretic peptide.

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We anticipate that there will be a dose response relationship between diesel exhaust inhalation and its pro-ischaemic effects. Measures of surrogate inflammatory,

ischaemic and cardiac markers will be used to determine the presence of adverse biochemical markers of myocardial ischaemia or injury. This will help determine the safe thresholds of diesel exhaust inhalation in patients with coronary heart disease.

8.2.4 Development of fibrinolytic and thrombotic models

We intend to explore whether the potential thrombogenic effects of air pollution can be further delineated using the Badimon chamber (BHF Project Grant PG/04/131; Dr Andrew Lucking). In brief, the Badimon chamber provides a powerful and elegant method of assessing *ex vivo* thrombus formation in an extracorporeal flow chamber [Badimon *et al*, 1999]. The technique utilises a continuous flow of venous effluent blood from the human forearm that is passed into a series of chambers containing a thrombogenic surface usually consisting of denuded porcine aortic strips. The characteristics of the flow chamber can be modified to generate low and high shear stress conditions that generate fibrin or platelet rich thrombi respectively. This powerful technique permits the dynamic formation of thrombus to be observed and allows the assessment of thrombotic and anti-thrombotic interventions. Combining the forearm model and Badimon chamber provides a unique opportunity to examine not only dynamic clot dissolution with endogenous t-PA release, but also to assess the prothrombotic effects of air pollution.

8.2.5 Assessment of new technologies

New strategies are currently in development to reduce diesel exhaust emissions, such as the introduction of new cleaner diesel fuels with ultra low sulphur content, and the retrofit of oxidation catalysts and particle filters. In the United States, the EPA is

subsidising a voluntary programme to retrofit particle traps on commercial and public heavy-duty diesel engines as part of the National Clean Diesel Campaign (<http://www.epa.gov/otaq/diesel/index.htm>). Whilst particle filtration technology can reduce emissions there are uncertainties about the regeneration of these filters and therefore their long-term efficacy. To date there have been no studies that address the potential for reducing particle emissions from diesel engines to prevent the adverse health effects of exposure to diesel exhaust.

Assessing the potential for new technologies to reduce emissions and prevent adverse cardiovascular effects will by necessity involve collaboration with industry. Through Professor Thomas Sandstrom at Umeå University we have access to a commercially available retrofit particle trap manufactured by Volvo (Gothenburg, Sweden). In future studies we will assess the efficacy of this particle trap and hope to conduct controlled exposures to diesel exhaust with and without particle filtration in both healthy volunteers and patients with coronary artery disease. These studies will determine the potential for commercially available technology to prevent the adverse vascular and pro-ischaemic effects of exposure to combustion-derived air pollution.

8.3 CLINICAL PERSPECTIVE

Using a unique exposure system we have demonstrated that healthy volunteers who inhale dilute diesel exhaust develop an impairment of two important, highly relevant and complementary aspects of vascular function: the regulation of vascular tone and endogenous fibrinolysis. We have extended these findings and have shown that brief exposure to dilute diesel exhaust promotes myocardial ischemia and inhibits endogenous fibrinolytic capacity in patients with stable asymptomatic coronary heart disease. Taken together our findings provide a plausible explanation for the epidemiologic observations that exposure to air pollution is associated with adverse cardiovascular events including acute myocardial infarction.

We believe environmental health policy interventions targeting reductions in the combustion component of urban air pollution should be considered in order to decrease the risk of adverse cardiovascular events. In future studies we hope to determine the minimum concentration of diesel exhaust exposure necessary to invoke these effects and assess the effects of commercially available retrofit exhaust treatments on cardiopulmonary health. These proposed studies will help inform environmental health policy and assist in setting safety thresholds for air quality standards.

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APPENDIX

PUBLICATIONS ARISING FROM THESIS

ORIGINAL ARTICLES

Mills NL, Törnqvist H, Robinson SD, Gonzales M, Darnley K, MacNee W, Boon NA, Donaldson K, Blomberg A, Sandström T, Newby DE. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. *Circulation* 2005;**112**:3930-3936. [Appended]

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